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ACTIVE SUBFRAGMENT OF AN ENDOGENOUS PEPTIDE

FIELD OF THE INVENTION

The invention relates to the field of angiogenesis, and more particularly to the use of a biologically active subfragment derived from the central region of human Histidine-Rich Glycoprotein (HRGP) as an inhibitor of angiogenesis. One of said subfragments is hereafter referred to as Pep2.

BACKGROUND

Angiogenesis, the formation of new capillaries from already existing blood vessels, is a procedure, which is essential during development and physiological conditions that require increased vascularisation, such as wound healing and the menstrual cycle. The resting vasculature is tightly regulated by a balance between pro- and anti-angiogenic factors. This balance is disturbed in a number of pathological processes, resulting in deficient angiogenesis, as in ischemic conditions, or excessive angiogenesis, as in rheumatoid arthritis, diabetic retinopathy and tumour growth. It has lately become well established that many types of tumours need to stimulate infiltration of new capillaries to grow and metastasise. Many interesting new therapies, based on pro- and anti-angiogenic substances, are therefore currently being tested in the clinic (Risau et al (1997), Carmeliet et al (2000), Folkman et al (1995, 2000), Hanahan et al (2000), Kerbel et al (2000)).

Histidine Rich Glycoprotein (HRGP) was identified by Heimburger et al in 1972. The protein is synthesized in the liver and has an unusual high content of Pro and His residues. These residues are predominantly situated in a His/Pro region in the centre of the protein and seem to be critical for its function. Until recently, though, very little has been known about the physiological role of HRGP. For a review, see Heimburger et al (1972), Koide et al (1986).

HRGP is present in human plasma at a concentration of approximately 100 µg/ml, which is considered to be very high. The amino acid sequence of mouse, rat, rabbit and human HRGP have been resolved and the protein seems to be well conserved among these vertebrate species (Drasin et al (1996), Hulett et al (2000), Borza et al (1996), Koide et al (1986)). See nucleotide and amino acid sequences of human HRGP under Genbank accession number NM000412.

After proteolytical cleavage from its 18 amino acid long signal peptide, mature HRGP can be divided into three main domains: The N-terminal domain, the His/Pro (central) domain and the C-terminal domain, all displaying different properties. Furthermore, the three domains are

suggested to be responsible for binding different ligands. The N-terminal domain contains two cysteine protease inhibitor (cystatin)-like stretches (Fig. 1A), which allows the classification of HRGP as a member of the cystatin superfamily together with α2HS glycoprotein, cystatin and kininogen. Whereas the central domain is very rich in proline and histidine residues resulting in e.g. the human form containing 12 more or less conserved tandem repeats of the pentapeptide HHPHG. In plasma, both the central domain and the C-terminal domain are disulfide bonded to the cystatin-like stretches in the N-terminal domain (Borza et al (1996)).

High molecular weight kininogen (HK) is structurally related to HRGP, possibly through a gene duplication, as the genes for HK and HRGP are located in close proximity on chromosome 3q. HK has been shown to interfere with endothelial cell function via a part of the protein with sequence similarities to the His/Pro-rich domain of HRGP (Zhang et al (2000)).

In general, HRGP binds a variety of different ligands, which can be divided into three major groups: ligands belonging to the coagulation/fibrinolysis system (e.g. heparin, plasminogen and fibrinogen), small ligands (e.g. heme and transition metal ions) and cells (e.g. T-cells, monocytes /macrophages) (Lamb-Wharton et al (1993), Olsen HM et al (1996)).

Furthermore, HGRP binds extracellular matrix components, such as trombospondin-1 (TSP-1) and vibronectin. Due to its binding to TSP-1, HRGP has previously been suggested to be proangiogenic, (Lijnen et al (1985)).

Juarez et al. (2002) reported that the His/Pro-rich domain of rabbit HRGP purified from plasma inhibits endothelial cell proliferation and vascularisation of matrigel plugs. Using recombinant HRGP, the data could even be extended to provide evidence for *in vivo* effects on tumour vascularisation. In a previous paper, HRGP was suggested to also, under certain circumstances, to promote angiogenesis and to attenuate the anti-angiogenic effect of TSP-1 by complex-formation between the two proteins. Juarez et al. suggest that this reported effect is dependent on contamination of the HRGP preparation by plasminogen, which could affect angiogenesis and TSP-1 indirectly.

Other examples of suggested functions of HRGP are modulation of fibrinogenesis, inhibition of insoluble immune complex formation and, recently, potentiation of the ingestion of apoptotic cells by macrophages (Kluszynski et al (1997), Gorgani et al (1997, 2002)).

In WO 02/076486, the inventors for the first time describe, the use of HRGP polypeptides, or its central regions, for the inhibition of angiogenesis. The application further shows methods for inhibiting angiogenesis by administering such a polypeptide to a mammal. Further disclosed are pharmaceutical compositions and articles of manufacture comprising HRGP polypeptides, antibodies and receptors that bind to an HRGP polypeptide, polynucleotides, vectors and host cells that encode HRGP polypeptides.

Later on, WO 02/064621 discloses a selection of HRGP polypeptides, or subfragments thereof, more particularly, specific, H/P-rich, repetitive pentapeptides from the central region of HRGP, as antiangiogenic. The subfragments are described as inhibitors of angiogenesis and are to be used for the treatment of diseases or conditions, in which angiogenesis is pathogenic. The compounds are predicted to have anti-tumour activity and are proposed to be of use in methods for inhibiting the growth of primary tumours or metastases. Also postulated are antibodies specific for the His-Pro rich domain of HRGP, as stimulators of angiogenesis for promoting neovascularisation in pertinent disease states.

None of the previous findings correctly specifies the actual active region, nor the minimal functional entity of HRGP, which for the first time has been identified and is described in the present invention. Having thus access to one or more minimal functional entities, a substantially shorter peptide can be used as a medicament. The advantages of using a shorter subfragment of HRGP are many. For ex. administration of long peptides is often associated with difficulties due to instability. Also, synthesis of longer peptides is often problematic, whereas shorter peptides are more convenient to synthesise. What is more, they are often less toxic due to higher specificity, which leads to less side effects.

SUMMARY OF THE INVENTION

The present invention relates to a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity and an amino acid length of between 25 and 145 amino acids. Said substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2) comprises amino acids corresponding to sequence number 330-364, or 383-390 of the mature HRGP protein (as listed in SEQ.ID.NO: 3), and is characterised by having antiangiogenic activity and an amino acid length of between 5 and 65aa.

In a presently preferred embodiment, said subfragment comprises approximately 35 amino acids, corresponding to sequence number 330-364 of full-length HRGP, as listed in amino acid sequence SEQ.ID.NO.1, and is hereafter referred to as Pep2.

In the present application, the inventors show that HRGP is proteolytically processed. The convincing results lead the inventors to suggest that human HRGP is naturally and cell/tissue/organ specifically proteolytically processed to render biologically active subfragments that have different biological functions. As proven herein for the first time, HIS5 thus corresponds to a naturally occurring consecutive subfragment of mature HRGP, comprising an anti-angiogenically active region of HRGP. What is more, when processed further, HIS5 can be cleaved to render Pep2, a further biologically active subfragment, corresponding to an equally anti-angiogenically active region of HRGP. In addition, Pep2 is in itself suggested to be a starting point for identifying a subsidiary minimal functional entity of HRGP, which is an inhibitor of angiogenesis.

Consequently, the invention also features a shorter consecutive subfragment of Pep2, corresponding at least to a part of Pep2, comprising about 5-35 amino acids, wherein the shorter subfragment still has anti-angiogenic activity. Alternatively, Pep2 can of course be extended either N-terminally or C-terminally, to render a longer consecutive subfragment, comprising at least part of Pep2, and wherein the longer subfragment still has anti-angiogenic activity.

Accordingly, the present invention in one embodiment comprises a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, for use as a medicament, and in another embodiment, the present invention relates to the use of said biologically active HRGP subfragment for the manufacture of a pharmaceutical composition for inhibiting angiogenesis in a mammal.

In this context, a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, can be produced recombinantly, synthetically, or be purified and/or isolated from plasma.

In a presently preferred embodiment, said substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, corresponds to SEQ.ID.NO:1, or an amino acid sequence which is at least 70% identical to SEQ.ID.NO:1, characterised by having anti-angiogenic activity. In an alternative embodiment, said subfragment of HRGP, which is an inhibitor of angiogenesis, corresponds to an amino acid being at least 75% homologous to SEQ.ID.NO:1.

Furthermore a multimer is envisioned, wherein the monomer corresponds to Pep2 or a substantially pure consecutive and biologically active subfragment of Pep2, which is an inhibitor of angiogenesis. Said multimer displays anti-angiogenic activity.

Also related to in the present context is a nucleic acid sequence encoding a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, and a vector comprising said nucleic acid sequence, as well as a host cell including said vector.

Furthermore, the invention relates to a pharmaceutical composition comprising a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, and optionally, an anti-angiogenic agent and/or an anti-neoplastic agent. Also, the composition may further comprise a pharmaceutical carrier acceptable for administration to a mammal.

Thus, the invention of course also relates to the use of a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, for the manufacture of a medicament for the treatment of an angiogenesis-related condition, such as, but not limited to, an angiogenesis-related condition, such as, but not limited to, cancer, myocardial angiogenesis, diabetic retinopathy, diabetic neovascularisation, inappropriate wound healing or an inflammatory disease. The invention also relates to a composition or a pharmaceutical composition comprising a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, as described above.

Included in the present invention is moreover a method for inhibiting angiogenesis in a mammal by administering a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, or a pharmaceutical composition as described above, to a mammal which may suffer from an angiogenesis-related condition such as, but not limited to, cancer, myocardial angiogenesis, diabetic retinopathy, diabetic neovascularisation, inappropriate wound healing or an inflammatory disease.

The invention is described in more detail in the following sections

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises an isolated or synthetically produced, substantially pure, consecutive, biologically active subfragment of human Histidine Rich Glycoprotein (HRGP) (see SEQ.ID.NO:3), which is an inhibitor of angiogenesis. Said substantially pure, biologically active subfragment is typically derived from the His/Pro rich region of HRGP (SEQ.ID.NO:2) and does in a most preferred embodiment correspond to amino acids 330-364 of full-length human HRGP, being referred to as Pep2 (SEQ.ID.NO:1). In another embodiment, the invention relates to a an isolated or synthetically produced, substantially pure, consecutive, biologically active subfragment of said Pep2, which is characterised as having anti-angiogenic properties.

In general, the His/Pro rich region of HRGP (SEQ.ID.NO:2) and Pep2 are considered a starting point for identifying a minimal functional entity of HRGP, which is further discussed below.

Until recently, the physiological function of HRGP has not been very well known. The present inventors were the first to suggest the involvement of HRGP in anti-angiogenesis (WO02/076486), although the details of its activity were still unknown.

In the present invention, the inventors show both *in vitro* and *in vivo* that HRGP, purified from human plasma, or recombinantly or synthetically produced, is a potent inhibitor of angiogenesis. As is shown in experiment 4, HRGP-treatment of fibrosarcoma-bearing mice resulted in decreased tumour angiogenesis and a more than 60% reduction in tumour volume compared to control-treated mice. The smaller tumour volume in HRGP-treated animals was not due to increased apoptosis of the tumour cells, in contrast to what has been reported from studies involving other inhibitors of angiogenesis, for instance TSP-1 and angiostatin. Instead, the proportion of proliferating tumour cells was significantly decreased in HRGP-treated animals, indicating a novel mechanism of action compared to other anti-angiogenic compounds. Most likely, the decreased proliferation reflects a limited supply of oxygen and nutrients to the tumour cells by the less extensive vasculature, compared to control-treated animals. (Auerbach et al 1991, Hawighorst et al (2002), O'Reilly et al (1996))

The plasma concentration of HRGP is relatively high, 100 µg/ml, already before treatment. The dose used in the tumour studies represents a 100% increase in the amount of HRGP in the treated mice every day, which could accumulate and reach substantially higher concentrations than normal. It is noteworthy in this context that it has not been possible to see accumulation of HRGP in the tumour tissue (data not shown). (Drasin et al (1996))

Without desire to limit the present invention, one plausible theory for the necessity of concentrations up to 100 µg/ml HRGP in plasma is that the protein is not active as a whole, but has to be processed into subfragments of which only some are anti-angiogenic.

Confirmative to the present finding, HRGP is sensitive to proteolytic cleavage and degradation products of the protein are often present in purified fractions, giving rise to a characteristic pattern of smaller bands, when resolved on polyacrylamide gels (Leung et al (1983), Hulett et al (2000), Kluszynski et al (1997)). Also, as is well known in the field of the art, HRGP binds a variety of endogenous substances, one of them being heparin. The separate domains interact with different ligands, probably due to the variety in function between the domains (Drasin et al (1996), Hulett et al (2000), Borza et al (1996), Koide et al (1986), Heimburger et al (1972))

As mentioned in a publication by Juarez et al (2002), HRGP has prior been suggested to comprise pro-angiogenic properties due to the binding to Trombospondin-1 (TSP-1), as it was thought to attenuate the anti-angiogenic properties of TSP-1. In the present application, though, the inventors show, that this is at least not the only function of HRGP. Moreover, presently the separate domains are shown to be responsible for different biological activities, one being anti-angiogenic, another being pro-angiogenic. Besides, the separate domains may be involved in other activities, not involving angiogenesis (Juarez et al (2002), Simantov et al (2001)).

Subsequently, HRGP is herein shown to acquire different properties during certain physiological circumstances. For instance, the heparin-binding affinity of HRGP is modulated and increased in the presence of Zn2+ and at low pH, a common environment e.g. in hypoxic tumours. Thus, Zn2+ is suggested to be an important cofactor for HRGP, or any antiangiogenic subfragment of HRGP, according to the present invention, in inhibiting angiogenesis. This is further evidenced by the fact that reduced Zn2+-content attenuated HRGP's inhibitory effect on tumour growth (data not shown).

Consequently, it is also clearly conceivable that a subfragment of HRGP, according to the present invention, which is generated in a specific milieu, and which under normal/healthy circumstances is not present in the plasma of the mammal, is responsible for the anti-angiogenic effects observed. This subfragment can e.g. be proteolytically cleaved from the full-length protein under conditions of increased angiogenesis, e.g. by proteolytic activity produced

in and/or secreted from a tumour. Consequently, said antiangiogenic subfragment can of course also be specifically enriched for during purification of the protein.

In the present application, the His/Pro region (listed as SEQ.ID.NO:2) is demonstrated to be the region responsible for the anti-angiogenic properties of HRGP. As shown in experiment 6B, the isolated His/Pro-rich domain of human HRGP inhibited chemotaxis as potently as the full-length protein. As the corresponding subfragment in rabbit HRGP can be produced by proteolytic processing of full-length HRGP by plasminogen, the inventors presume that this does also apply to human HRGP, since immunoblotting of a purified fraction of full length human HRGP, with an antibody directed against the His/Pro-rich domain, recognises a subfragment corresponding in size to the recombinantly produced His/Pro-rich domain. Surprisingly, the truncated version His 4 (as shown in experiment 6A) did not inhibit endothelial cell chemotaxis, even though it covers the complete His/Pro-rich domain. The reason for this is most probably that an improper folding of the protein prevents the active site of the His/Pro-rich domain from being correctly presented.

Furthermore, a 25 amino acid long subfragment of the His/Pro-rich region, located in direct succession to Pep2, and corresponding to amino acids 365-389 of mature human HRGP (SEQ.ID.NO:4) was clearly demonstrated not to be active for inhibiting angiogenesis, as shown in experiment 9. This further strengthens the hypothesis of a minimal functional entity of HRGP, not corresponding to the whole part of the His/Pro region, but rather to a shorter fragment such as Pep2.

Accordingly, the present inventors could convincingly show that the His/Pro region comprises an active part of the protein, and that a cell, tissue, or organ specific proteolytic cleavage of the full-length protein during, e.g. angiogenesis, inflammation, or in/or in the vicinity of a tumour, most probably leads to activation of at least one of HRGP's antiangiogenic properties, either by facilitating the exposure of a previously hidden active site/region of said central region, or by releasing one or more minimum active fragment(s) carrying said same or another active site/region.

The potent inhibitory effect of a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, on tumour growth, most likely mediated via decreased tumour angiogenesis, provides an interesting target for further studies. Also, the use of such a small, naturally occurring fragment for anti-angiogenesis as a clinical strategy to treat inflammatory diseases, e.g. rheumatoid arthritis, is attractive in many ways. Since anti-angiogenic therapy directly targets vasculature engaged in active angiogenesis, the risk of unwanted side effects is low, which is even lowered by elimiting the potential side effects of a longer peptide, including e.g. pro-angiogenetic properties. Also, problems with general toxicity are minimized when using therapies based on naturally occurring small proteins. Finally, making use of a body's own

response to a specific trait of e.g. tumour cells, namely the naturally expressed and/or secreted proteases that in a sick mammal proteolytically process HRGP to said active subfragments, in itself guarantees an approach to treating a disease that will be less toxic and less hampered by undesired side effects, because the therapy mimics and enhances the hosts natural defence instead of introducing a foreign factor.

Consequently, a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, as a biologically active, shorter fragment of HRGP, is a much-preferred peptide for use as a medicament in angiogenesis-dependent disease states, as it is less likely to be toxic and less likely to induce side effects due to higher specificity.

The present inventors successfully demonstrate the anti-angiogenic activity of Pep2 in an animal model, as shown in experiment number 10, followed by a dose-response study confirming the *in vitro* results pointing towards a saturation effect of said Pep2, shown in experiment number 8, thus suggesting a preferable dose for administration.

In the present context, the term "HRGP" refers to the mature human HRGP peptide, which comprises about 507 amino acid residues and which corresponds to SEQ.ID.NO:3. The mature human HRGP is comprised in the 525 aa-long full-length HRGP, as listed in SEQ.ID.NO:15 (see also NM000412) and including an 18aa-long signal peptide, which is cleaved of in the mature peptide.

A "subfragment", refers to a subfragment of variable size, which is typically derived from the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis.

"Pep2" refers to an amino acid subfragment of 35 amino acids (SEQ.ID.NO:1), which is derived from the His/Pro region (SEQ.ID.NO:2) of HRGP and which correspond to SEQ.ID.NO:1.

"Pep3" refers to an amino acid fragment of 25 amino acid residues corresponding SEQ.ID.NO:4, which is derived from the His/Pro region of HRGP and has further been described in W002/064621.

A "His-Peptide" is an amino acid fragment, comprising approximately 25 histidine residues, which in one embodiment corresponds to SEQ.ID.NO:5. Further, "His1" corresponds to SEQ.ID.NO:6, "His2" corresponds to SEQ.ID.NO:7, "His3" to SEQ.ID.NO:8, "His4" to SEQ:ID.NO:9 and finally, "His5" corresponds to SEQ.ID.NO:10. The results displaying the ability of inducing chemotaxis of these truncated versions of HRGP are shown and further discussed in experiment number 7.

Sequence identities referred to in the present invention are described below:

SEQ.ID.NO:1 Pep2

(348-382 (-18 signalpeptiden)) 330-364

DLHPHKHHSHEQHPHGHHPHAHHPHEHDTHRQHPH

35aa

SEQ.ID.NO:2 central region of human HRGP

(258-408 (-18 signalpeptiden)) 240-390

HLGHPFHWGGHERSSTTKPPFKPHGSRDHHHPHKPHEHGPPPPPDERDHSHGPPLPQGPPPLLPMSCSSCQ
HATFGTNGAQRHSHNNNSSDLHPHKHHSHEQHPHGHHPHAHHPHEHDTHRQHPHGHHPHGHHPHGHHP
HGHHPHGHHPHC

151aa

SEQ.ID.NO:3: mature human HRGP

(1-525 (-18 signalpeptiden)) 1-507

VSPTDCSAVEPEAEKALDLINKRRRDGYLFQLLRIADAHLDRVENTTVYYLVLDVQESDCSVLSRKYWNDCEP
PDSRRPSEIVIGQCKVIATRHSHESQDLRVIDFNCTTSSVSSALANTKDSPVLIDFFEDTERYRKQANKALEK
YKEENDDFASFRVDRIERVARVRGGEGTGYFVDFSVRNCPRHHFPRHPNVFGFCRADLFYDVEALDLESPKN
LVINCEVFDPQEHENINGVPP**HLGHP**FHWGGHERSSTTKPPFKPHGSRDHHHPHKPHEHGPPPPPDERDHS
HGPPLPQGPPPLLPMSCSSCQHATFGTNGAQRHSHNNNSSDLHPHKHHSHEQHPHGHHPHAHHPHEHDT
HRQHPHGHHPHGHHPHGHHPHGHH**PHGHHPHC**HDFQDYGPCDPPPHNQGHCCHGHGPPPGHLRRRGP
GKGPRPFHCRQIGSVYRLPPLRKGEVLPLPEANFPSFPLPHHKHPLKPDNQPFPQSVSESCPGKFKSGFPQVS
MFFTHTFPK

508

SEQ.ID.NO:4 Pep3

(383-407 (-18 signalpeptiden)) 365-389

GHHPHGHHPHGHHPHGHHPHGHHPH

25aa

SEQ.ID.NO:5 HISpep

HHHHHHHHHHHHHHHHHHHHHHHHH

25aa

SEQ.ID.NO:6 HIS2

(19-258 (-18 signalpeptiden)) 1-240

VSPTDCSAVEPEAEKALDLINKRRRDGYLFQLLRIADAHLDRVENTTVYYLVLDVQESDCSVLSRKYWNDCEP
PDSRRPSEIVIGQCKVIATRHSHESQDLRVIDFNCTTSSVSSALANTKDSPVLIDFFEDTERYRKQANKALEK
YKEENDDFASFRVDRIERVARVRGGEGTGYFVDFSVRNCPRHHFPRHPNVFGFCRADLFYDVEALDLESPKN
LVINCEVFDPQEHENINGVPP**H**

SEQ.ID.NO:7 HIS3

(19-338 (-18 signalpeptiden)) 1-320

VSPTDCSAVEPEAEKALDLINKRRRDGYLFQLLRIADAHLDRVENTTVYYLVLDVQESDCSVLSRKYWNDCEP
PDSRRPSEIVIGQCKVIATRHSHESQDLRVIDFNCTTSSVSSALANTKDSPVLIDFFEDTERYRKQANKALEK
YKEENDDFASFRVDRIERVARVRGGEGTGYFVDFSVRNCPRHHFPRHPNVFGFCRADLFYDVEALDLESPKN
LVINCEVFDPQEHENINGVPP**HLGHP**FHWGGHERSSTTKPPFKPHGSRDHHHPHKPHEHGPPPPPDERDHS
HGPPLPQGPPPLLPMSCSSCQHATFGTNGAQ

SEQ.ID.NO:8 HIS4

(19-408 (-18 signalpeptiden)) 1-390

VSPTDCSAVEPEAEKALDLINKRRRDGYLFQLLRIADAHLDRVENTTVYYLVLDVQESDCSVLSRKYWNDCEP
PDSRRPSEIVIGQCKVIATRHSHESQDLRVIDFNCTTSSVSSALANTKDSPVLIDFFEDTERYRKQANKALEK
YKEENDDFASFRVDRIERVARVRGGEGTGYFVDFSVRNCPRHHFPRHPNVFGFCRADLFYDVEALDLESPKN
LVINCEVFDPQEHENINGVPP**HLGHP**FHWGGHERSSTTKPPFKPHGSRDHHHPHKPHEHGPPPPPDERDHS
HGPPLPQGPPPLLPMSCSSCQHATFGTNGAQRHSHNNNSSDLHPHKHHSHEQHPHGHHPHAHHPHEHDT
HRQHPHGHHPHGHHPHGHHPHGHH**PHGHHPHC**

SEQ.ID.NO:9 HIS5

(258-408 (-18 signalpeptiden)) 240-390

HLGHPFHWGGHERSSTTKPPFKPHGSRDHHHPHKPHEHGPPPPPDERDHSHGPPLPQGPPPLLPMSCSSC
QHATFGTNGAQRHSHNNNSSDLHPHKHHSHEQHPHGHHPHAHHPHEHDTHRQHPHGHHPHGHHPHGHH
PHGHH**PHGHHPHC**

SEQ.ID.NO:10 Pep4

(408-432 (-18 signalpeptiden)) 390-414

CHDFQDYGPCDPPPHNQGHCCHGHG

SEQ.ID.NO:11 Pep5

(432-457 (-18 signalpeptiden)) 414-439

GPPPGHLRRRGPGKGPRPFHCRQIGS

SEQ.ID.NO:12 Pep6

(458-493 (-18 signalpeptiden)) 440-475

VYRLPPLRKGEVLPLPEANFPSFPLPHHKHPLKPDN

SEQ.ID.NO:13 Pep7

(494-525 (-18 signalpeptiden)) 476-507

QPFPQSVSESCPGKFKSGFPQVSMFFTHTFPK

SEQ.ID.NO:15: full-length human HRGP including an 18aa signalsequence

1-525

MKALIAALLLITLQYSCAVSPTDCSAVEPEAEKALDLINKRRRDGYLFQLLRIADAHLDRVENTTVYYLVLD
VQESDCSVLSRKYWNDCEPPDSRRPSEIVIGQCKVIATRHSHESQDLRVIDFNCTTSSVSSALANTKDSPVLI
DFFEDTERYRKQANKALEKYKEENDDFASFRVDRIERVARVRGGEGTGYFVDFSVRNCPRHHFPRHPNVFGF
CRADLFYDVEALDLESPKNLVINCEVFDPQEHENINGVPP**HLGHP**FHWGGHERSSTTKPPFKPHGSRDHHH
PHKPHEHGPPPPPDERDHSHGPPLPQGPPPLLPMSCSSCQHATFGTNGAQRHSHNNNSSDLHPHKHHSHE
QHPHGHHPHAHHPHEHDTHRQHPHGHHPHGHHPHGHHPHGHH**PHGHHPHC**HDFQDYGPCDPPPHNQG
HCCHGHGPPPGHLRRRGPGKGPRPFHCRQIGSVYRLPPLRKGEVLPLPEANFPSFPLPHHKHPLKPDNQPFPQ
SVSESCPGKFKSGFPQVSMFFTHTFPK
525aa

In a general aspect of the present invention, a consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, is preferably selected from a 25 to 145 amino acid residues long peptide, such as from an at least 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, or at least 145 amino acid residues long peptide. Such a consecutive subfragment does not correspond to pep3.

In another, presently preferred embodiment, a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), comprises amino acids corresponding to sequence number 330-364, or 383-390 of mature HRGPSaid subfragments are characterised in having antiangiogenic activity and an amino acid length of between 5 and 65, such as between 25 and 50, 10 and 25, 10 and 30, 20 and 35, 25 and 35, or between 20 and 40 amino acids. Such a subfragment does at least comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, or 65 consecutive amino acids.

In a presently most preferred embodiment, though, a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), comprises amino acids corresponding to sequence number 330-364, as listed in amino acid sequence SEQ.ID.NO.1, and has an amino acid length of between 5 and 35 amino acids, such as at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, or 35 amino acids.

Other typical embodiments of the invention, are peptides or a fragment of a peptide corresponding to one of the amino acid sequences as listed in the accompanying sequence listing as SEQ.ID.NO. 11-14. Pep4-Pep7

A consecutive amino acid fragment, in the present context, relates to a sequence of amino acids, which is derived successively and uninterrupted from the original amino acid sequence, to which it correlates. In particular, Pep2 thus corresponds to a naturally occurring succession of amino acids of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), namely to amino acids 330-364 of full length human HRGP, as listed in amino acid sequence SEQ.ID.NO.1.

Additionally, any conservative variant of the sequence of a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, is by virtue of its functional relationship to said subfragment considered to be inside the scope of the present invention.

A conservative variant of a sequence is in the present context defined as an amino acid sequence which is conserved at least 70%, such as 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, when comparing variants of the same amino acid sequence between different species. The degree of conservation of a variant can, as is well known in the field, be calculated according to its derivation of PAM (see Dayhoff, Schwartz, and Orcutt (1978)), or based on comparisons of Blocks of sequences derived from the Blocks database as described by Henikoff and Henikoff (1992).

Conservative substitutions may be made, for example according to table 1 below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

Table 1

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

Such replacements may also be made by unnatural amino acids include; alpha* and alpha-disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, ß-alanine*, L-a-amino butyric acid*, L-g-amino butyric acid*, L-a-amino isobutyric acid*, L-e-amino caproic acid#, 7-amino heptanoic acid*, L-methionine sulfone#*, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline#, L-thioproline*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)#, L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid # and L-Phe (4-benzyl)*. The notation * is herein utilised to indicate the hydrophobic nature of the derivative whereas #

is utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or b-alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, which will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the a-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, see for example, Simon RJ et al, (1992) and Horwell DC et al (1995).

Peptides and/or subfragments of the invention may be in a substantially isolated form. It will be understood that the peptide/amino acid sequence may be mixed with carriers or diluents, which will not interfere with the intended purpose of the peptide/amino acid sequence and still be regarded as substantially isolated. A peptide/ amino acid sequence of the invention may also be in a substantially purified form, in which case it will generally comprise the peptide/amino acid sequence or a fragment thereof in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a peptide of the invention.

Furthermore, any amino acid sequence being at least 70% identical, such as being at least 72%, 75%, 77%, 80%, 82%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical with the amino acid sequence of a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, is also considered to be inside the scope of the present invention.

By a polypeptide having an amino acid sequence at least, for example 95% identical to a reference amino acid sequence, is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the amino acid sequence may include up to 5 point mutations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence: up to 5% of the amino acids in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acids in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

In the present invention, a local algorithm program is best suited to determine identity. Local algorithm programs, such as (Smith-Waterman) compare a subsequence in one sequence with a subsequence in a second sequence, and find the combination of subsequences and the alignment of those subsequences, which yields the highest overall similarity score. Internal gaps, if allowed, are penalized. Local algorithms work well for comparing two multidomain proteins, which have a single domain, or just a binding site in common.

Methods to determine identity and similarity are codified in publicly available programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J et al (1994)) BLASTP, BLASTN, and FASTA (Altschul, S.F. et al (1990)). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S.F. et al, Altschul, S.F. et al (1990)). Each sequence analysis program has a default scoring matrix and default gap penalties. In general, a molecular biologist would be expected to use the default settings established by the software program used.

The advantages of using a smaller subfragment of HRGP, such as Pep2 for the treatment of angiogenesis-related diseases are obvious. For example, smaller peptide subfragments are more convenient for the development of a pharmaceutical. Longer peptides are considered difficult to handle, as they are often unstable and readily degraded once distributed in the body. Further, a shorter peptide is also considered to have fewer side effects, as the likelihood of the shorter peptide to interact with other biologically active substances is lesser than for a longer peptide.

As used herein, a "pro-angiogenic activity" or "pro-angiogenic property" is defined as an activity, which promotes angiogenesis. An amino acid sequence with pro-angiogenic activity promotes angiogenesis by interaction with other factors involved in the process leading to angiogenesis, or by direct action in specific subprocesses during angiogenesis such as, but not limited to, chemotaxis. Furthermore, the pro-angiogenic activity is characterized in that it will promote angiogenesis in naturally occurring physiological processes such as embryonic growth, wound healing, and in pathological processes such as rheumatoid arthritis, systemic lupus erythematosus, arteriosclerosis and cancer. Examples of pro-angiogenic factors are fibroblast growth factors, Follistatin, G-CSF, IL-8, TGF-α, TGF-β etc

As used herein, an amino acid sequence/peptide, which displays an "anti-angiogenic activity" or "anti-angiogenic property" is defined as displaying an activity, which inhibits angiogenesis. The anti-angiogenic an amino acid sequence/peptide inhibits angiogenesis by interaction with other factors involved in the process leading to angiogenesis or by direct action in specific subprocesses during angiogenesis such as, bit not limited to, chemotaxis. Furthermore, the anti-angiogenic activity is characterised in that it will inhibit and decrease angiogenesis in naturally occurring physiological processes such as embryonic growth, wound healing, and in pathological processes such as rheumatoid arthritis, systemic lupus erythematosus,

arteriosclerosis and cancer. Examples of anti-angiogenic factors are Angiostatin, Angioarrestin, Endostatin, Fibronectin subfragment, Cartilage-derived inhibitor etc

As described in the present invention, subfragments of HRGP, such as Pep2 and subfragments thereof, are in their natural environment produced by a "naturally occurring proteolytical event", characterised in that the polypeptide is cleaved by endogenous enzymes (e.g. proteases) to generate subfragments of which some display biological activity such as being involved in the angiogenesis process. In one embodiment of the present invention, a subfragment may comprise anti-angiogenic properties; in another embodiment a subfragments may comprise pro-angiogenic properties. Furthermore, other subfragments may comprise properties, which do not affect the process of angiogenesis.

Accordingly, as previously described, the naturally occurring subfragments of HRGP, are suggested to comprise different physiological functions. E.g. in a previous publication, it has been suggested that HRGP interacts with Trombospondin-1 (TSP-1). It is further suggested that the interaction between TSP-1 and HRGP attenuates the anti-angiogenic properties of TSP-1, thereby proposing a pro-angiogenic role of HRGP. (Juarez et al (2002))

In the present invention, convincing results have been presented, which on the contrary support the importance of HRGP as an angiogenesis inhibitor. These contradictory results strengthen the hypothesis that the different subfragments of HRGP comprise different physiological properties. It is in the present invention further shown, that HRGP in its natural environment most frequently occur as a subfragmented protein. Thus, is it suggested that some of the subfragments derived from HRGP, preferably derived from the His-Pro region, and preferably again, derived from Pep2, comprise the anti-angiogenic activity of HRGP, which is silenced in the unprocessed mature peptide.

A "biologically active subfragment" in the present context, refers to a subfragment, which is active in its biological environment, which is characterised by that it maintains its biological activity when removed from its natural environment. A biologically active subfragment is further characterised in that it is not dependent upon any other stimulants or co-factors to function, but is active in it self. However, it should be mentioned, that a biologically active fragment of HRGP might behave differently depending on the physiological environment such as the presence of Zn^{2+} and a specific pH.

Another aspect of the present invention includes isolated polynucleotide sequences encoding a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis. Such polynucleotides include, but are not limited to, mRNAs, cDNAs and genomic DNAs, as well as diagnostically or therapeutically useful fragments thereof. Also included are variants of such polynucleotides, which variants encode for a fragment, derivative or analogue of a substantially pure consecutive

subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis.

Isolated nucleic acid molecules of the invention can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding an HRGP polypeptide, but free of sequences that normally flank one or both sides of the wild-type gene in a naturally occurring genome. The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring nucleic acid sequence since such non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome.

An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include an engineered nucleic acid such as a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

Isolated nucleic acids within the scope of the invention can be obtained using any method including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, PCR techniques can be used to obtain an isolated nucleic acid containing a nucleic acid sequence sharing identity with art known sequences of HRGP.

PCR refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in "PCR Primer: A Laboratory Manual," Dieffenbach et al (1995). When using RNA as a source of template, reverse transcriptase can be used to synthesize complimentary DNA (cDNA) strands.

Isolated nucleic acids of the invention also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long

oligonucleotides (e.g., >100 nucleotides) can be synthesised that contain the desired sequence, with each pair containing a short segment of complementary (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Isolated nucleic acids of the invention also can be obtained by mutagenesis. For example, an isolated nucleic acid that shares identity with an in the art known HRGP sequence can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and substitutions, as well as combinations of deletions, insertions, and substitutions.

In addition, nucleic acid and amino acid databases (e.g., GenBank®) can be used to obtain an isolated polynucleotide within the scope of the invention. For example, a sequence having homology to a nucleic acid sequence encoding an in the art known HRGP subfragment or an amino acid sequence having homology to an in the art known HRGP subfragment amino acid sequence can be used as a query to search GenBank®. Examples of nucleic acids encoding known HRGP polypeptides include the following GenBank® Accession Nos.: NM 000412 (human); AF194028 (mouse); AF 194029 (rat); and U32189 (rabbit).

Furthermore, nucleic acid hybridisation techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, a nucleic acid sequence encoding a HRGP subfragment can be used as a probe to identify a similar nucleic acid by hybridisation under conditions of moderate to high stringency. Moderately stringent hybridisation conditions include hybridisation at about 42°C in a hybridisation solution containing 25 mM KPO_4 (pH 7.4)@ 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 5 0% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5xI0^7$ cpm/µg), and wash steps at about 50°C with a wash solution containing 2X SSC and 0.1% SDS. For high stringency, the same hybridisation conditions can be used, but washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% SDS.

Once a nucleic acid is identified, the nucleic acid then can be purified, sequenced, and analysed to determine whether it is within the scope of the invention as described herein.

Hybridisation can be done by Southern or Northern analysis to identify a DNA or RNA sequences, respectively that hybridises to a probe. The probe can be labelled with biotin, digoxygenin, an enzyme, or a radioisotope such as ^{32}P or ^{35}S . The DNA or RNA to be analysed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridised with the probe using standard techniques well known in the art. See, for example, sections 7.39-7.52 of Sambrook et al (1989).

The present invention also includes vectors comprising isolated polynucleotides encoding HRGP subfragments, such as Pep2, and host cells comprising these vectors. The vectors of the present invention may include, for example, plasmids, recombinant viruses, cloning vectors, or expression vectors. Host cells may be genetically engineered (transduced or transformed or transfected) with the vectors of this invention. The engineered host cells can be selected from a broad variety such as from plant cells, or eukaryotic cells, such as mammal, insect or yeast cells, or procaryotic cells, such as bacterial cells. Said host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the 25 genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The term "expression vector" or "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter I 0 operably linked with the polypeptide coding sequence; and, optionally, with other sequences, e.g., transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, e.g., enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, Gene Expression Technology. Methods in Enzyniology, vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises anucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (e.g., a cell membrane, a viral lipid envelope, etc.). Vectors include, but are not limited to replicons (e.g., RNA replicons, bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous selfreplicating circular or linear DNA or RNA, e.g., plasmids, viruses, and the like (U.S. Patent No. 5,217,879),

A wide variety of methods are known to the person skilled in the art which can be used to obtain a substantially pure subfragment of HRGP, or a polypeptide derived thereof, as previously described. For example, intact or subfragmented HRGP can be purified from freshly collected human plasma by chromatography on phosphocellulose in the presence of proteinase inhibitors following the procedures Kluszynski et al (1997), Rylatt et al (1981)

As shown in experiment 2 the resultant protein is approximately 99 % pure, as estimated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Many materials can be used as a source to obtain a substantially pure polypeptide, for example tissue culture cells expressing the particular polypeptide of interest can be used to obtain a substantially pure polypeptide. Furthermore, in addition to polypeptide purification techniques such as affinity chromatography and HPLC, other polypeptide synthesis techniques can of course be used to produce the sought after polypeptide.

Subfragments of HRGP, such as Pep2, may also be produced by recombinant methods. The cDNA sequences of rabbit (Borza et al. (1996)), rat, mouse (Hulett et al. (2000)) and human (Koide et al.(1986)) HRGP are art known. Methods for the expression of a protein product from a cDNA clone are well known. See, for example, Maniatis et al. (1989). A subfragment of HRGP produced can be either Pep2 or a subfragment thereof. Suitable expression systems include, without limitation, microorganisms such as bacteria (for example, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors; yeast (for example, *Saccharomyces* and *Pichia*) transformed with recombinant yeast expression vectors; insect cell systems infected with recombinant virus expression vectors (for example, baculovirus); plant cell systems infected with recombinant virus expression vectors (for example, cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (for example, Ti plasmid) containing fusion protein nucleotide sequences; or mammalian cell systems (for example, HEK, COS, CHO, BHK, 293, VERO, HeLa, MDCK, W138, and NIH 3T3 cells) transformed with expression constructs containing promoters derived from the genome of mammalian cells (for example, the metallothionein promoter) or from mammalian viruses (for example, the adenovirus late promoter and the vaccinia virus 7.5K promoter). Also useful as host cells are primary or secondary cells obtained directly from a mammal, transfected with a plasmid vector or infected with a viral vector such as herpes viruses, retroviruses, vaccinia viruses, attenuated vaccinia viruses, canary pox viruses, adenoviruses, adeno-associated viruses, lentiviruses and herpes viruses, among others.

Furthermore, synthetic production of HRGP peptides, or any other peptide mentioned in the present invention, is defined as an event were amino acids are attached together to generate a peptide/protein during a chemical procedure. This may be performed according to standard protein synthesis procedures.

For diagnostic applications, a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, may be labelled with a detectable moiety. This detectable moiety is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as H^3, C^{14}, P^{32}, S^{35}, or I^{125} ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as

alkaline phosphatase, betagalactosidase or horseradish peroxidase. Any method known in the art for conjugating a polypeptide to a detectable moiety may be employed. See, for example, Hunter et al. (1962); David et al. (1974); Pain et al. (1981); and Nygren et al. (1982).

A substantially pure consecutive subfragment comprised in the present invention, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, may also be coupled to a detectable moiety useful for *in vivo* imaging to image areas of neovascularisation. The HRGP subfragment may be labelled with a detectable moiety such as a radio-opaque agent or radioisotope and administered to a host, preferably into the bloodstream, and the presence and location of the labelled HRGP subfragment is assayed. The HRGP subfragment may be labelled with any moiety that is detectable in a host, whether by nuclear magnetic resonance, radiology, or other detection means known in the art. Radioisotope can be, for example, ^{186}Re, ^{188}Re, ^{64}Cu, ^{67}Cu, ^{212}Bi, ^{123}I, ^{131}I, ^{211}At, ^{177}Lu, ^{47}SC, ^{105}Rh, ^{109}Pd, ^{153}Sm, ^{199}Au, ^{99m}Tc, ^{111}In, ^{124}I, ^{18}F, ^{11}C, ^{198}AU, ^{75}Br, ^{76}Br, ^{77}Br, ^{13}N, ^{34m}CI, ^{38}CI, ^{52m}Mn, ^{55}Co, ^{62}Cu, ^{68}Ga, ^{72}As, ^{76}As, ^{72}Se, ^{73}Se, or ^{71}Se.

A substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, coupled to a toxin may be used as a therapeutic agent to target a receptor. Such a subfragment, may be coupled to any toxic polypeptide that mediates a cytotoxic effect within the cytoplasm of a cell by procedures well known in the art. Preferred toxic polypeptides include ribosome inactivating proteins, e.g., plant toxins such as an A chain toxin (e.g., ricin A chain), saporin, bryodin, gelonin, abrin, or pokeweed antiviral protein (PAP), fungal toxins such as α-sarcin, aspergillin, or restrictocin, bacterial toxins such as diphtheria toxin (DT) or Pseudomonas exotoxin A, or a ribonuclease such as placental ribonuclease or angiogenin. Other useful toxic polypeptides are the pro-apoptotic polypeptides, e.g., Bax, Bad, Bak, Bim, Bik, Bok, or Hrk. Furthermore, more than one functional fragment (e.g. 2, 3, 4, 6, 8, 10, 15, or 20) of one or more (e.g., 2, 3, 4, or 6) toxins can be coupled to a subfragment of HRGP. Where repeats are included, they can be immediately adjacent to each other, separated by one or more targeting fragments, or separated by a linker peptide as described above. The invention also includes functional fragments of any of these polypeptides coupled to a HRGP subfragment.

The present invention furthermore discloses antibodies comprising specific binding affinity for a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis. Antibodies having specific binding affinity for such subfragments, can be produced through standard methods. As used herein, the terms "antibody" or "antibodies" include intact molecules as well as fragments thereof that are capable of binding to an epitopic determinant in such a HRGP subfragment. The term "epitope" refers to an antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains, and typically have specific three

dimensional structural characteristics, as well as specific charge characteristics. Epitopes generally have at least five contiguous amino acids.

The terms "antibody" and "antibodies" include polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain Fv antibody fragments, Fab fragments, and F(ab)2 fragments. Monoclonal antibodies are particularly useful.

In general, a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, is produced recombinantly, by chemical synthesis, or by purification of the native protein, and then used to immunize animals.
Various host animals including, for example, rabbits, chickens, mice, guinea pigs, and rats, can be immunised by injection of a subfragment of interest. Adjuvants can be used to increase the immunological response depending on the host species, and include Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface-active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin (KLH), and dinitrophenol. Polyclonal antibodies are heterogeneous populations of antibody molecules that are specific for a particular antigen, which are contained in the sera of the immunised animals. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular epitope contained within an antigen, can be prepared using standard hybridoma technology. See Kohler et al.(1975). In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture, such as the human B-cell hybridoma technique (See Kosbor et al. (1983) and Cole et al. (1983)) or the EBV-hybridoma technique (See Cole et al. (1983)). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridoma producing the monoclonal antibodies of the invention can be cultivated in vitro or in vivo.

A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Chimeric antibodies can be produced through standard techniques.

Humanised forms of the murine antibodies may be made by substituting the complementarity determining regions of the mouse antibody into a human frameworkdomain, by methods known in the art. Selected murine framework residues also may be substituted into the human recipient immunoglobulin. Monoclonal antibodies with a desired binding specificity can be commercially humanised (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals are also features of the invention. See, for example, Green et al. (1994), U.S. Patent 5,545,806 and U.S. Patent 5,569,825.

Antibody fragments that have specific binding affinity for a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, can be generated by known techniques. For example, such fragments include, but are not limited to, F(ab')2 fragments that can be produced by pepsin digestion of the antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')2 fragments.

Alternatively, Fab expression libraries can be constructed. See, for example, Huse et al (1989). Single chain Fv antibody fragments are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge (e.g., 15 to 18 amino acids), resulting in a single chain polypeptide. Single chain Fv antibody fragments can be produced through standard techniques. See, for example, U.S. Patent No. 4,946,778.

Once produced, antibodies or fragments thereof are tested for recognition of HRGP subfragments by standard immunoassay methods including, for example, ELISA techniques or RIA. See, Short Protocols in Molecular Biology (1992). Suitable antibodies preferably have equal binding affinities for recombinant and native proteins.

The HRGP subfragment antibodies of the current invention can be packaged in a diagnostic kit comprising at least one HRGP subfragment-specific antibody described herein, which may be conveniently used to detect a HRGP subfragment in a sample for research or diagnostic purposes.

Antibodies to a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. See Zola, Monoclonal Antibodies: A Manual of Techniques (1987).

Competitive binding assays rely on the ability of a labelled standard (which may be a HRGP subfragment or an immunologically reactive portion thereof) to compete with the test sample analyte (HRGP) for binding with a limited amount of antibody. The amount of HRGP in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilised before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte, which remain unbound.

The subfragment HRGP antibodies of the current invention can be neutralising antibodies, capable of substantially inhibiting or eliminating a biological activity of a subfragment of HRGP,

such as the antiangiogenic activity of a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, as assayed in experiment number 9. The HRGP subfragment antibodies of the current invention can also be agonistic or antagonistic of angiogenesis.

The invention further encompasses a method for identifying receptors specific for a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, and the receptor molecules identified and isolated thereof. Using techniques well known in the art, HRGP subfragments, such as Pep2, may be employed to develop affinity columns for the isolation of a receptor from cell lysates. Isolation of a receptor is followed by amino acid sequencing. From this amino acid sequence information, polyR nucleotide probes can be developed for use in cloning polynucleotide sequences that encode a receptor.

A substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, can be used as a birth control agent by reducing or preventing the uterine vascularisation required for embryo implantation. In such a method of birth control, an amount of a HRGP subfragment sufficient to prevent embryo implantation is administered to a female mammal. In one aspect of the birth control method, an amount of a HRGP subfragment sufficient to block embryo implantation is administered before or after intercourse and fertilization have occurred, thus providing an effective method for birth control, possibly a "morning after" method. Administration methods may include, but are not limited to, pills, injections (intravenous, subcutaneous, intramuscular), suppositories, vaginal sponges, vaginal tampons, and intrauterine devices.

Plasma depleted of biologically active HRGP subfragments may be prepared using anti-subfragment HRGP antibodies, such as anti-Pep2 antibodies, in various in the art known immunopurification techniques. Such techniques include, but are not limited to, immunoprecipitation, immunoaffinity bead purification and immunoaffinity column chromatography. HRGP subfragments-depleted plasma may also be prepared by passing plasma over a Nicolumn prepared with Ni-NTA agarose resin (Qiagen Inc., Chatsworth, USA), by methods well known in the art.

A typical composition of the present invention comprises a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis. Furthermore, in some embodiments of the invention, the composition also includes an anti-angiogenic agent and/or an anti-neoplastic agent. In some embodiments, the composition may also include a pharmaceutical carrier.

An anti-angiogenic agent may e.g. be selected from, but is not limited to, interferon-inducible protein 10 and subfragments and analogues of interferon-inducible protein 10, TGF-β, thrombospondin, IL-1 (interleukin), IFN-γ (interferon), IFN-α, tissue inhibitor of metalloproteinase-1 (TIMP-1), platelet factor 4 (PF4), protamine, fumagillin, angiostatin and the like.

Furthermore, an anti-neoplastic agent may be included in the composition. Such an anti-neoplastic agent may be a chemotherapeutic agent toxic to tumour cells. Examples of such are alkylating agents, antimetabolites, natural products, hormones and antagonists, biological response modifiers (such as interferon and hematopoietic growth factors), differentiating agents such as butyrate derivatives, antibodies to tumour agents and other miscellaneous agents. Other examples include taxol, cyclophosphamide, carboplatinum, cisplatinum, cisplatin, gancyclovir, campothecin, paclitaxel, hydroxyurea, 5-acacytidine, 5-aza-2′-deoxycytidine, suramin, retinoids and the like.

Furthermore, the combination therapy with a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, is not limited to an anti-angiogenic agent or an anti-neoplastic agent, but may also include combination treatment methods using an anti-inflammatory agent such as prednisone, a cox-2 inhibitor, and the like. Suitable inflammatory agents may also include ibuprofen and aspirin.

Pharmaceutical compositions comprising a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, may be administered by intravenous infusion, or may be injected subcutaneously, intramuscularly, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, intrapulmonarily, intratumorally or intralesionally.

The dosage depends on physiological factors such as age, weight, the nature of the patients' illness, the patients' sex, etc. The dosage further depends on the route of administration, the nature of the formulation, other drugs being administered and the judgement of the attending physician.

Said patient is selected from the group comprising a mammal, such as human, ape, dog, or rabbit.

A pharmaceutically acceptable carrier is e.g. a biologically compatible vehicle suitable for administration to a mammalian subject. Such pharmaceutically acceptable compositions

typically contain from about 0.1 to 90% (such as 1-20% or 1-10%) by weight of a therapeutic agent of the invention in a pharmaceutically acceptable carrier.

Injectable formulations of the composition may contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol and the like).

For intravenous injections, water-soluble versions of the compounds may be administered by a drip method, whereby a pharmaceutical formulation containing HRGP and a physiological expedient is fused. The physiological acceptable carriers may include, but is not limited to, 5% dextrose, 0,9% saline, Ringer's solution or any other suitable carriers.

Furthermore, intramuscular preparations can be dissolved and administered in a pharmaceutical excipient such as 0.9% saline or 5% glucose solution.

For a topical administration, a semi-solid ointment formulation typically contains a formulation of the active ingredient from about 1 to 20% e.g. 5 to 10%, in a carrier such as a pharmaceutical cream base. Formulations for topical use include, but is not limited to, creams, solutions, tinctures, drops, lotions and ointment which comprise the active ingredient and various supports and vehicles. The optimal percentage of the therapeutic agent in each pharmaceutical formulation varies according to the formulation itself and the therapeutic effect, which is desired in the specific pathologies and correlated therapeutic regiments. Methods of making such formulations can be found in "Remingtons Pharmaceutical Sciences"

An insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such as an ester of a long acid fatty chain (e.g. ethyl oleate).

The amount of a pharmaceutical that is desirable to produce a medical effect in a treated mammal (e.g. human) is specified as a "therapeutically effective amount". Accordingly, the dosage for any patient depends on many factors, including the patients age, sex, body surface area, the particular compound to be administered, the patients' general health, route of administration, and also other drugs being administered concurrently. The preferred administration will most likely be intravenous.

In one embodiment, a substantially pure consecutive subfragment of the central region of human histidin rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, will be administered to a patient who is suffering from a disease state were it is desired to inhibit angiogenesis. In another embodiment, a pro-angiogenic subfragment thereof may be administered to a patient who is suffering from a condition were it is desired to promote angiogenesis.

Appropriate treatment subjects include, without limitation, those patients suffering from an angiogenesis-related condition, such as for example angiogenesis-related cancers rhadbomyosarcoma, glioblastoma multiforme, leiomyosarcoma, prostate carcinoma, mammary carcinoma, lung carcinoma, melanoma, bladder carcinoma, pancreatic carcinoma and renal carcinoma, were a treatment with an anti-angiogenic agent is preferable.

Furthermore, patients suffering from an angiogenesis related condition such as, but not limited to, diabetic retinopathy, diabetic neovascularization, retrolental fibroplasias, trachoma, neovascular glaucoma, psoriasis, angio-fibromas, immune and non-immune inflammation, capillary formation within atherosclerosis plaques, myocardial angiogenesis, hemangiomas, excessive wound repair, various inflammatory diseases and any other disease characterised by excessive and/or deregulated angiogenesis, may benefit from a treatment with a substantially pure consecutive subfragment of the central region of human histidin rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis.

FIGURE LEGENDS

Fig. 1. Human HRGP inhibits CAM angiogenesis.

A. Structurally HRGP can be divided into three main domains; the N-terminus with two cystatin-like stretches, a histidine-proline-rich (His/Pro-rich) middle domain and the C-terminus. Both the His/Pro-rich middle domain and the C-terminus are disulfide bonded to the N-terminal part of the protein.

B. HRGP was obtained from three different sources; either purified from human plasma (pHRGP) or recombinantly produced, with or without a His-tag (His-HRGP and rHRGP, respectively). Both Coomassie staining and Western blot (WB) with an HRGP-specific antibody revealed, except for the full length protein at approximately 75 kDa, a number of smaller subfragments.

C. FGF-induced angiogenesis in the chicken CAM was effectively inhibited in the presence of HRGP.

Fig. 2. HRGP inhibits tumour growth and vascularization.

Mice bearing palpable fibrosarcoma tumours were treated daily with s.c. injections of either pHRGP or PBS (A) or His-HRGP, human IgG (B) and the tumour volume was measured daily with a caliper. Endostatin (ES) was included in one study for comparison (B). C. Staining for CD31 on paraffin-sections of the tumours from mice treated with either IgG (ctrl) or HRGP. Arrows indicate vessels. D. Stereological quantification of vascular parameters in three tumours from IgG- (ctrl) or HRGP-treated mice. Individual values from each tumour is indicated with a circle.

Fig. 3. Decreased proliferation in tumours from HRGP-treated mice.

Paraffin-sections of tumours from HRGP- or IgG (ctrl)-treated mice were stained for A. apoptotic (TUNEL-positive) and B. proliferating (Ki67-positive) cells. The proportions are expressed as number of positively stained cells per total number of cells counted. * denotes $P < 0.05$.

Fig. 4. HRGP inhibits chemotaxis of primary endothelial cells.

A. Chemotaxis of primary BCE cells induced by FGF-2 (F) was completely arrested in the presence of 100 ng/ml HRGP (H).

B. The same concentration of HRGP also inhibited VEGF-A (V)-induced chemotaxis.

C. However, FCS-induced chemotaxis of BCE cells was not inhibited in the presence of HRGP.

D. PCI (P) had no effect on FGF-2-induced chemotaxis of BCE cells.

E. FGF-2-induced chemotaxis of NIH 3T3 murine fibroblasts was not inhibited by HRGP.

Fig. 5. The His/Pro-rich domain of HRGP inhibits chemotaxis.

A. Schematic overview of HRGP and truncated versions (His 2-5). The ability to inhibit FGF-2-induced chemotaxis of endothelial cell is indicated by +/-.

B. Quantification of the inhibitory effect of His 5 on FGF-2 induced chemotaxis of endothelial cells.

C. Western blot (WB) of mature HRGP using the peptide antibodies 0115, 0116 and 0119 gives different patterns of immunoreactivity (10% SDS-PAGE). Western blot of the subfragment His 5 using antibody 0119 yields immunoreactivity with one peptide migrating around 30 kDa (12.5% SDS-PAGE).

Fig. 6: Dose response study of Pep2

Fig. 7: Comparative study Pep2/Pep3

Fig. 8: Animal study of Pep2

Fig. 9: Adhesion assay

EXPERIMENTS

Experiment 1: Tissue culture

Procedure

The porcine aortic endothelial (PAE) cell line overexpressing FGF receptor-1 (FGFR-1) (Wennström et al 1992) was cultured in Ham's F12/10% fetal bovine serum (FCS) and NIH 3T3 murine fibroblasts were cultured in Dulbecco's modified medium (DMEM)/10% newborn calf serum (NCS). F12, DMEM and serum were bought from Life Technologies. Primary bovine adrenal cortex capillary endothelial (BCE) cells were cultured on gelatin-coated dishes in DMEM/10% NCS and 2 ng/ml FGF-2 (Boehringer Mannheim). For chemotaxis assays, both PAE and NIH 3T3 cells were serum-starved over night in 0.1% BSA and BCE cells in 0.5% NCS. Human embryonic kidney (HEK) 293-EBNA cells were cultured in DMEM/10% FCS. Approximately every second month they were given 0.25 mg/ml G418 (Calbiochem) to ensure positive selection of the EBNA-1 expressing cells.

Experiment 2: HRGP expression vectors, transfection and purification of protein

HRGP from three different sources were used in this study; HRGP purified from human plasma (pHRGP) and recombinant HRGP produced in HEK-293-EBNA cells, with or without a His-tag (His-HRGP and rHRGP, respectively).

Procedure

Full length cDNA encoding human HRGP including the signal sequence (amino acid residues 1-18) was cloned into the pCEP-Pu2 (Vernersson et al 2002) expression vector. Expression vectors for His-tagged HRGP variants were also constructed using the same vector. The truncations were produced by PCR-amplification of shorter parts of the protein. N-terminally of the HRGP coding region, a His-tag (six histidine residues) was added to enable purification. An enterokinase cleavage site was introduced between the His-tag and the HRGP coding region, to allow removal of the His-tag. In these vectors, the HRGP signal sequence was excluded and instead, the PCR-product was ligated in frame with the BM40 signal sequence in pCEP-Pu2.

HEK 293-EBNA cells were used to produce the recombinant HRGP. These cells are stably transfected with the EBNA-1 gene, which is also expressed by the pCEP-Pu2 vector, to efficiently prevent chromosomal integration of transfected plasmid DNA, resulting in an overall high yield of recombinant protein, without the need for characterization of individual clones. The HRGP expression vectors were transfected into the 293-EBNA cell line using Lipofectamine™ (Invitrogen) and selected with 2.5 mg/ml puromycin (Sigma). To avoid contamination of bovine HRGP in the conditioned medium, a defined serum-replacement medium, TCM™ (ICN Biomedicals) was used instead of FCS.

Chromatography on phosphocellulose (Kluszinsky et al 1997, Rylatt et al 1981) in the presence of proteinase inhibitors was used to purify HRGP from freshly collected human plasma (pHRGP), or recombinant untagged HRGP from conditioned medium (rHRGP). His-tagged HRGP was purified using Ni-NTA agarose (Qiagen) according to the manufacturer's protocol. Protein-containing fractions were pooled and dialysed against PBS pH 7.4.

Results

Recombinant untagged HRGP migrates with an apparent lower molecular mass on SDS-PAGE than HRGP purified from plasma (Fig. 1B), probably reflecting different levels of glycosylation. This difference in migration rate between endogenous and recombinant protein is not apparent when comparing pHRGP and His-HRGP (Fig. 1B), due to the addition of 10 extra amino acid residues to His-HRGP (providing a His-tag and enterokinase cleavage site to enable removal of the tag). Western blotting with an HRGP-specific antibody, revealed apart from the full-length protein, a number of smaller subfragments in all three purified fractions (Fig. 1B). These subfragments are most likely degradation products of HRGP, since mass-spectrometry analysis failed to reveal any contaminating peptides.

Experiment 3: HRGP inhibits CAM angiogenesis

The effect of HRGP on angiogenesis was tested in the chicken chorioallantoic membrane (CAM) assay.

Procedure

The conditions for the chorioallantoic membrane (CAM) assay followed essentially a previously described procedure (Friedlander et al (1995), Dixelius et al, (2000)). FGF-2 (Boehringer Mannheim) and VEGF-A (Peprotech) was used at 0.2 mg per filter and purified pHRGP or rHRGP at 3 mg per filter. The treated CAM's were inspected in a light microscope and the score, from 0 (low) to 3 (high) was based on the number of vessel branch points. Average values for 5-6 embryos were recorded.

Results

A filter disc soaked in vehicle or growth factor, with or without HRGP, was placed on top of the CAM in the vicinity of a large vessel and incubated for three days. Treatment of the CAM with growth factors, such as FGF-2, stimulated angiogenesis (Fig. 1C; Table 1). Application of FGF-2 or VEGF-A together with pHRGP on the CAM led to an efficient suppression of newly formed vessels, whereas pre-established vessels remained unaffected during these conditions. The same result was obtained using rHRGP (data not shown).

Experiment 4: Animal studies: HRGP inhibits tumour growth in mice due to decreased proliferation

Angiogenesis plays an important role in the growth of aggressive tumours, therefore the effect of HRGP on fibrosarcoma growth in mice was investigated, using both pHRGP (Fig. 2A) and His-HRGP (Fig. 2B). To determine the mechanism whereby HRGP treatment reduced tumour growth, the proportion of apoptotic and proliferating cells in tumour tissue from the two treatment groups was further quantified.

Procedure

Animal work was carried out at the local animal facility and was approved by the Uppsala University board of animal experimentation and thus performed according to the UKCCCR guidelines for the welfare of animals in experimental neoplasia (Workman et al (1988)). The mice were anesthetized with isoflurane (Forene; Abbott) during all manipulations. Female, 5 week old, C57BL6/J mice (Mollegard/Bomhultgard, Denmark) were injected with 0.5 x 106 T241 fibrosarcoma cells subcutaneously (s.c.) into the left flank. Animals carrying palpable tumours were randomized (n = 7-10 animals/treatment group) and received treatment with vehicle (PBS), 4 mg/kg/day of pHRGP or 5 mg/kg/day of human IgG or His-HRGP, given as daily subcutaneous injections in the right flank. The tumours were measured with a caliper once a day, in a blind procedure, and volumes were calculated by the formula P/6 x width2 x length. When the tumours reached the upper limit of 2 cm^3, the mice were sacrificed. Three animals in each group were perfused with 4% paraformaldehyde in PBS pH 7.4. Tumours were embedded in paraffin according to standard histological procedures and sectioned at 4 mm thickness.

Results

C57/bl6 mice were inoculated subcutaneously with T241 fibrosarcoma cells on their left flank and when the tumours were palpable (after 4-6 days), a daily treatment was initiated with HRGP or control (PBS; Fig. 2A, or human IgG; Fig. 2B). Endostatin was included in one study for comparison (Fig. 2B). The treatment was given as subcutaneous injections in the right flank until the size of the control tumours reached the upper limit of 2 cm^2 (11 days; Fig. 2A, 14 days; Fig. 2B). Injections with both pHRGP (4 mg/kg/day) and His-HRGP (5 mg/kg/day) led to a drastic reduction in tumour growth and at the time of sacrifice, the size of the tumours was reduced by 67% (Fig. 2A) and 61% (Fig. 2B) respectively, compared to control-treated animals. Endostatin treatment (25 mg/kg/day) lacked significant inhibitory effect on tumour growth in this model (32% decrease).

Apoptotic cells were visualized by TUNEL staining. No difference in tumour apoptosis could be found between the two groups (Fig. 3A) and the percentage of apoptotic cells in the tumours was generally low (less than 1%) in both groups. The proportion of proliferating cells was determined by immunohistochemical staining for the KI67 antigen. As shown in figure 3B, there were significantly less proliferating cells in the tumours from HRGP-treated mice compared to those from control-treated animals; 29% and 43% respectively.

Experiment 5: Reduced vascularization in tumours from HRGP-treated mice

Mice treated with HRGP show a reduced vascularization pattern in tumours, as shown in an immunostaining of paraffin-section of tumours.

Procedure

Paraffin-sections of tumours from control- or HRGP-treated animals were immunohistochemically (IHC) stained for CD31 and KI67 using a goat anti-mouse CD31 antibody (1506; Santa Cruz), diluted 1:500 and incubated at +4°C over night, and a rat anti-mouse KI67 antibody (TEC-3/M7249; DAKO) diluted 1:50 and incubated for 30 min. at room temperature, according to standard IHC procedures. Detection of apoptotic cells by the TUNEL-technique was performed using ApopTag™ (Intergen Company) according to the manufacturer's protocol. Stereological quantification of vascular parameters was performed as described earlier by Weibel et al (1979) and Gundersen et al (1988). Quantification of the relative number of Tunel- and KI67-positive cells was calculated from approximately 3000 cells per tumour section, in three tumours from each treatment group. Significance at the level of $P < 0.05$ was calculated using Students t-test.

Results

Sections of paraffin-embedded tumours from control (IgG)- or HRGP-treated mice were immunohistochemically stained for CD31 expression to visualise the vessels. Visual inspection revealed clear changes in the extent of vascularisation of the treated tumours compared to control, in particular with regard to the reduced vessel diameter in response to HRGP treatment (Fig. 2C). Stereological quantification of vascular parameters was performed as described earlier by Weibel et al (1979) and Gundersen et al (1988). This method of quantifying tumour angiogenesis relates the length, volume and surface area of the vessels to tumour volume. The results show that all six vascular parameters determined were reduced in tumours from HRGP-treated mice (Fig. 2D).

Experiment 6 A: HRGP inhibits chemotaxis of primary endothelial cells

HRGP acts through the inhibition by chemotaxis of endothelial cells, which is a common feature of anti-angiogenic molecules.

Procedure

The migration assay was performed using a modified Boyden chamber as described earlier (Wassberg et al, (1999)), using micropore nitrocellulose filters (8 mm pore) coated with type-1 collagen solution (BCE) at 100 mg/ml (Vitrogen 100, Collagen Corp) or fibronectin solution (PAE/FGFR-1, NIH 3T3) at 50 mg/ml (BD Biosciences). BCE, PAE/FGFR-1 or NIH 3T3 cells were preincubated or not with HRGP (100 ng/ml) for 30 min, trypsinised and resuspended at a concentration of 5.0 x 105 cells/ml. FGF-2 and VEGF-A was used at 5 ng/ml, HRGP and the protein C inhibitor (PCI) at 100 ng/ml and FCS at 1%. After 4 h at 37°C, migrated cells were stained with Giemsa and counted using an image analysis software (Easy Image Analysis) from

Tekno Optik AB, Sweden. All samples were analysed in at least six wells for each treatment and at several separate occasions.

Preparation and Zn2+-loading of peptides

Peptides were bought from Innovagen AB, 22 370 Lund, Sweden and delivered dried in glass vials. Peptides were weighed out and dissolved in phosphate buffered saline to a concentration of 1 mg/ml. A solution of 10 mM ZnCl2 in destilled H20 was prepared. Peptides were incubated with a 10-fold molar excess of the peptide for 30 min, or alternatively, ZnCl2 to a final concentration of 5 mM was included in the cell suspension added to both wells of the Boyden chamber assay, as described above.

Results

A common feature of the anti-angiogenic molecules described to date is their ability to inhibit chemotaxis of endothelial cells in vitro. In accordance, inclusion of 100 ng/ml of rHRGP completely blocked FGF-2-induced chemotaxis of BCE cells as shown in figure 4A. The same concentration of rHRGP also attenuated VEGF-A-induced chemotaxis (Fig. 4B). However, chemotaxis induced by FCS was not inhibited by rHRGP (Fig. 4C). To demonstrate specificity, another plasma protein of approximately the same mass as HRGP, the protein C inhibitor (PCI), was included for comparison. PCI had no effect on FGF-2-induced chemotaxis (Fig. 4D). The specificity of HRGP towards endothelial cells was implied by the fact that FGF-2-induced chemotaxis of NIH 3T3 murine fibroblasts was not affected by the presence of rHRGP (Fig. 4E).

Experiment 6B: The His/Pro-rich domain of HRGP inhibits chemotaxis

Procedure/results

To determine which part of HRGP that was responsible for the anti-angiogenic effect, recombinant truncated forms of the protein was produced (Fig. 5A). Truncated versions containing the C-terminal part of HRGP, but lacking an intact N-terminus, were not possible to produce, maybe due to instability. Of the four truncated proteins tested (His 2-5), only the His/Pro-rich domain (His 5) inhibited chemotaxis of endothelial cells towards FGF-2 (see +/- indications in Fig. 5A).

Experiment 7: The His/Pro-rich domain as a naturally occurring degradation product of HRGP

HRGP is proteolytically cleaved from full-length protein to smaller subfragments when purified from plasma. One subfragment from the His/Pro region is suggested to comprise the full activity of the protein (minimal functional entity), this could be a naturally occurring subfragment.

Procedure

Purified HRGP was separated on 10% (pHRGP, rHRGP and His-HRGP) or 12.5% SDS-PAGE (His 5). The monoclonal mouse anti-human HRGP antibody MO37 (Takara) was used at 0.05 mg/ml

and the rabbit polyclonal peptide antibodies 0115, 0116 and 0119 were used at a 1:5000 dilution. For detection of His-tagged proteins an anti-penta His antibody, directly conjugated to HRP (34460; Qiagen) was used at 1:5000 dilution.

Results

Domain-specific antibodies against HRGP were raised by immunizing rabbits with three different peptides; 0115, 0116 and 0119. The positions of these peptides in the HRGP protein are indicated in figure 5A. Western blot of mature HRGPwith the three peptide-antibodies revealed apart from the full length protein, a specific pattern of reactivity with smaller HRGP-derived subfragments (Fig. 5B). Interestingly, the apparent molecular weight of 30 kDa of the His/Pro-rich subfragment (His 5) equals that of a naturally occurring HRGP subfragment recognized by the antibody 0119, directed against the His/Pro-rich domain of HRGP (Fig. 5B). This indicates the possibility that the His/Pro-rich domain in human HRGP may be proteolytically released from the full-length protein *in vivo*.

Experiment 8: Dose response study Pep2

Chemotaxis: Primary endothelial cells were starved over night in 0.5% normal calf serum. The migration assay was performed using a modified Boyden chamber, using micropore nitrocellulose filters (8 mm pore) coated with type-1 collagen solution at 100 microg/ml (Vitrogen 100, Collagen Corp). Cells were trypsinised and resuspended at 7.5 x 105 cells/ml. The cell suspension was added in the upper chamber and FGF-2 (10 ng/ml), HRGP (100 ng/ml), peptide 2 or 3 (50 ng/ml) in medium containing 0.25% FCS were added to the lower chamber. The peptides were dissolved in PBS containing a 10-fold molar excess (Zn:peptide) of Zn^{2+}-acetate. After 6 h at 37°C, cells that had migrated through the filter were stained with Giemsa and counted using an the Easy Image Analysis software. All samples were analysed in at least six wells for each treatment and at several separate occasions.

Experiment 9: Comparative study pep2/pep3: chemotaxis

Primary endothelial cells were starved over night in 0.5% normal calf serum. The migration assay was performed using a modified Boyden chamber, using micropore nitrocellulose filters (8 mm pore) coated with type-1 collagen solution at 100 microg/ml (Vitrogen 100, Collagen Corp). Cells were trypsinized and resuspended at 7.5 x 105 cells/ml. The cell suspension was added in the upper chamber and FGF-2 (10 ng/ml), HRGP (100 ng/ml), peptide 2 or 3 (50 ng/ml) in medium containing 0.25% FCS were added to the lower chamber. The peptides were dissolved in PBS containing a 10-fold molar excess (Zn:peptide) of Zn2+-acetate. After 6 h at 37°C, cells that had migrated through the filter were stained with Giemsa and counted using an the Easy Image Analysis software. All samples were analyzed in at least six wells for each treatment and at several separate occasions.

Experiment 10: Animal study Pep2

Procedure

Tumour study: Female, 5 week old, C57BL6/J mice (Mollegard/Bomhultgard, Denmark) were injected with 0.5 x 106 T241 fibrosarcoma cells subcutaneously (s.c.) into the left flank. Animals carrying palpable tumours were randomised (n = 5-10 animals/treatment group) and received treatment with vehicle (Zn-acetate in NaCl), 5 mg/kg/day of HIS1 (=full-length HRGP) or Pep2, in a volume of 100 microliters, given as s.c. injections daily, in the right flank. The tumours were measured with a caliper once a day, in a blind procedure, and volumes were calculated by the formula P/6 x width2 x length. When the tumours reached the upper limit of 2 cm^3, the mice were sacrificed. The animals were anesthetised with isoflurane (Forene; Abbott) during all manipulations.

Experiment 10: Adhesion:

BCE cells were detached using non-enzymatic "Cell Dissociation Solution" (Sigma), washed and re-suspended in DMEM/0.1% BSA and FGF-2 (2 ng/ml). The cells were seeded into wells pre-coated with BSA, collagen I, collagen IV, laminin-1 or vitronectin, respectively (CytoMatrix ECM 205 kit, Chemicon). The cells were incubated for 45 (collagen I, collagen IV and vitronectin) or 60 minutes (laminin-1), washed three times in PBS and stained with Hoechst 33342 (1 µg/ml; Molecular Probes). The cells were micro-photographed using the 2x objective and the number of attached cells was counted using the "Easy Image Analysis" software (Tekno Optik). Statistical analysis was performed by ANOVA and Tukeys honestly significant difference (HSD) test. The standard deviation is based on the pooled variance.

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CLAIMS

1. A substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity and an amino acid length of between 25 and 145 amino acids.

2. A substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), comprising amino acids corresponding to sequence number 330-364, or 383-390, characterised in having antiangiogenic activity and an amino acid length of between 5 and 65, such as between 25 and 50, or between 20 and 40 amino acids.

3. A substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), comprising amino acids corresponding to sequence number 330-364, as listed in amino acid sequence SEQ.ID.NO.1, characterised in having antiangiogenic activity and an amino acid length of between 5 and 35 amino acids.

4. A substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2) corresponding to amino acid sequence SEQ.ID.NO.1, characterised in having antiangiogenic activity.

5. A substantially pure consecutive subfragment of amino acid sequence SEQ.ID.NO.1, characterised in having antiangiogenic activity and an amino acid length of between 5 and 25 amino acids.

6. A substantially pure consecutive subfragment of amino acid sequence SEQ.ID.NO.1, characterised in having antiangiogenic activity and an amino acid length of between 5 and 15 amino acids.

7. A substantially pure consecutive subfragment of amino acid sequence SEQ.ID.NO.1, characterised in having antiangiogenic activity and an amino acid length of between 5 and 10 amino acids.

8. A subfragment according to any of claims 1-7, being isolated from human histidine rich glycoprotein (HRGP).

9. A subfragment according to any of claims 1-7, being isolated from proteolytically processed human histidine rich glycoprotein (HRGP) purified from plasma.

10. A subfragment according to any of claims 1-7, being recombinantly produced, or isolated from recombinantly produced human histidine rich glycoprotein (HRGP).

11. A synthetically produced subfragment according to any of claims 1-7.

12. A subfragment according to any of the preceding claims, characterised in that it does not bind to thrombospondin.

13. A subfragment according to any of the preceding claims, characterised in that it does not promote angiogenesis.

14. An antiangiogenic pharmaceutical composition comprising an effective amount of a substantially pure polypeptide corresponding to any of the preceding claims.

15. An antiangiogenic pharmaceutical composition according to claim 14, further comprising a pharmaceutically acceptable carrier.

16. An antiangiogenic pharmaceutical composition according to any of claims 14-15, further comprising an anti-angiogenic agent.

17. An antiangiogenic pharmaceutical composition according to claim 16, wherein said anti-angiogenic agent is selected from the group consisting of angiostatin, thrombostatin, endostatin, interferon-α, interferon-inducible factor 10, and platelet factor 4.

18. An antiangiogenic pharmaceutical composition according to any of claims 14-17, further comprising an anti-neoplastic agent.

19. An antiangiogenic pharmaceutical composition according to claim18, wherein said anti-neoplastic agent is selected from the group consisting of taxol, cyclophosphamide, carboplatinum, cisplatinum, cisplatin, gancyclovir, camptothecin, paclitaxel, hydroxyurea, 5-azacytidine, 5-aza-2'-deoxycytidine, and suramin.

20. An antiangiogenic pharmaceutical composition according to any of claims 14-19, further comprising an anti-inflammatory agent.

21. An antiangiogenic pharmaceutical composition according to claim 20, wherein said anti-inflammatory agent is selected from the group consisting of prednisone, a cox-2 inhibitor, ibuprofen and aspirin.

22. An antiangiogenic pharmaceutical composition according to any of claims 14-21, further comprising an effective amount of Zn^{2+}.

23. A substantially pure consecutive subfragment, according to any of claims 1-13 for use as a medicament.

24. A substantially pure consecutive subfragment, according to any of claims 1-13 for use as a medicament for the inhibition of angiogenesis in a mammal.

25. A substantially pure consecutive subfragment, according to any of claims 1-13 for use as a medicament for inhibiting tumour growth in a mammal.

26. A substantially pure consecutive subfragment, according to any of claims 1-13 for use as a medicament for treating and/or inhibiting cancer in a mammal.

27. A substantially pure consecutive subfragment, according to any of claims 1-13 for use as a medicament for treating and/or inhibiting myocardial angiogenesis, diabetic retinopathy, diabetic neovascularization, inappropriate wound healing, or an inflammatory disease.

28. Use of a substantially pure consecutive subfragment, according to any of claims 1-13 for the manufacture of a pharmaceutical composition for the inhibition of angiogenesis in a mammal.

29. Use of a substantially pure consecutive subfragment, according to any of claims 1-13 for the manufacture of a pharmaceutical composition for treating and/or preventing cancer in a mammal.

30. Use of a substantially pure consecutive subfragment, according to any of claims 1-13 for the manufacture of a pharmaceutical composition for inhibiting tumour growth in a mammal.

31. Use of a substantially pure consecutive subfragment, according to any of claims 1-13 for the manufacture of a pharmaceutical composition for treating and/or inhibiting myocardial angiogenesis, diabetic retinopathy, diabetic neovascularization, inappropriate wound healing, or an inflammatory disease in a mammal.

32. Use according to any of claims 28-31, wherein said mammal is a mouse.

33. Use according to any of claims 28-31, wherein said mammal is a rat.

34. Use according to any of claims 28-31, wherein said mammal is a human.

35. Method for inhibiting angiogenesis in a mammal, comprising administering a pharmaceutical composition according to any of claims 14-22 to a mammal in need thereof.

36. An isolated nucleic acid that encodes a consecutive subfragment, according to any of claims 1-13.

37 An expression vector comprising a nucleic acid sequence according to claim 36, optionally operatively linked to a promoter and/or additional regulatory sequences that regulate the expression of said nucleic acid sequence in a eukaryotic or prokaryotic host cell.

38. A host cell transformed and/or transfected with an expression vector according to claim 37.

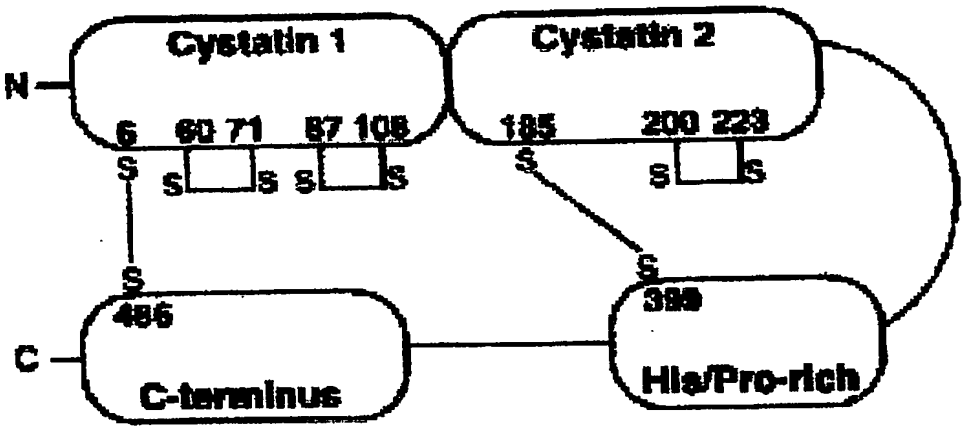
39. A host cell according to claim 38 selected from the group consisting of mammalian cells, such as human, mouse or rat cells, and bacteria, yeast, or insect cells.

40. Method for inhibiting angiogenesis in a mammal, comprising administering an isolated nucleic acid, a host cell, and/or a vector according to any of claims 36 or 39 to a mammal in need thereof.

ABSTRACT

The present invention relates to one or more new biologically active subfragments, localized to the His/Pro region of Histidine Rich Glycoprotein (HRGP) and the use of these subfragments. One of the active subfragments is referred to as Pep2. Pep2 is claimed for use as a medicament. The invention further includes a method for the inhibition of angiogenesis by administration of said subfragment and to the patient, and to the use of a substantially pure consecutive subfragment of the central region of human histidine rich glycoprotein (HRGP) (SEQ.ID.NO: 2), characterised in having antiangiogenic activity, such as Pep2, or a subfragment of Pep2, which is an inhibitor of angiogenesis, for the manufacture of a pharmaceutical composition for inhibiting angiogenesis. The invention also includes pharmaceutical compositions and articles of manufacture of said subfragment, e.g. Pep2. The invention further comprise antibodies, and receptors that bind to Pep2, as well as Pep2 polynucleotides, vectors encoding Pep2.

A:


Cystatin 1
Cystatin 2
N
6
60 71
87 108
185
200 223
S
S
S
S
S
S
S
S
486
C-terminus
399
His/Pro-rich
C

B:

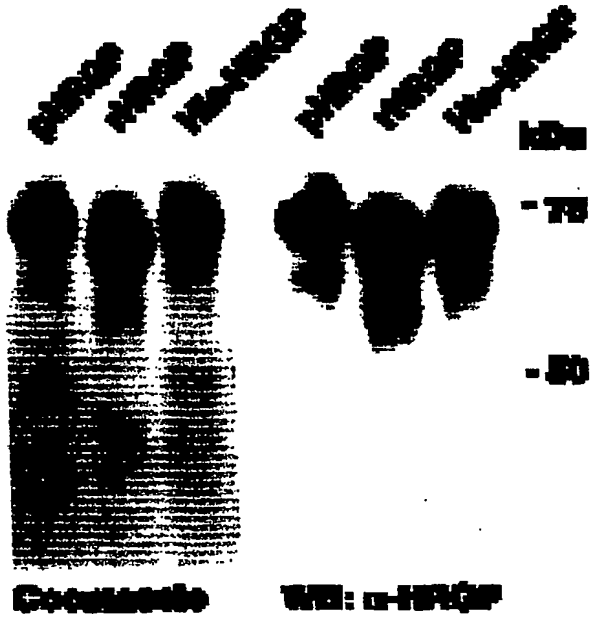

kDa
-75
-50

Fig 1

C:

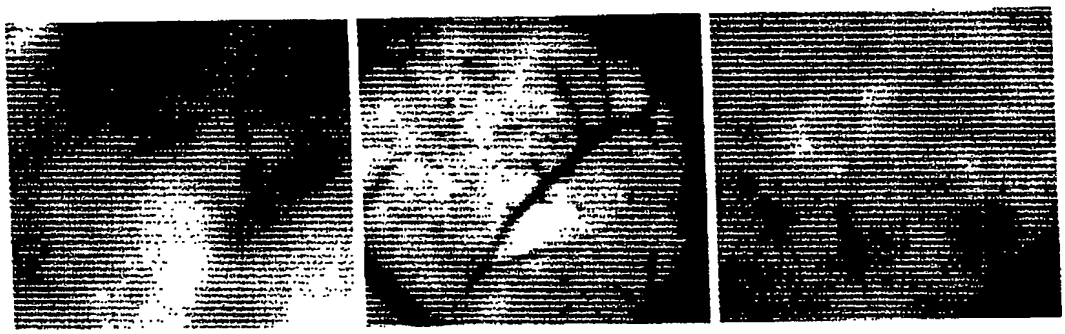


Fig 1

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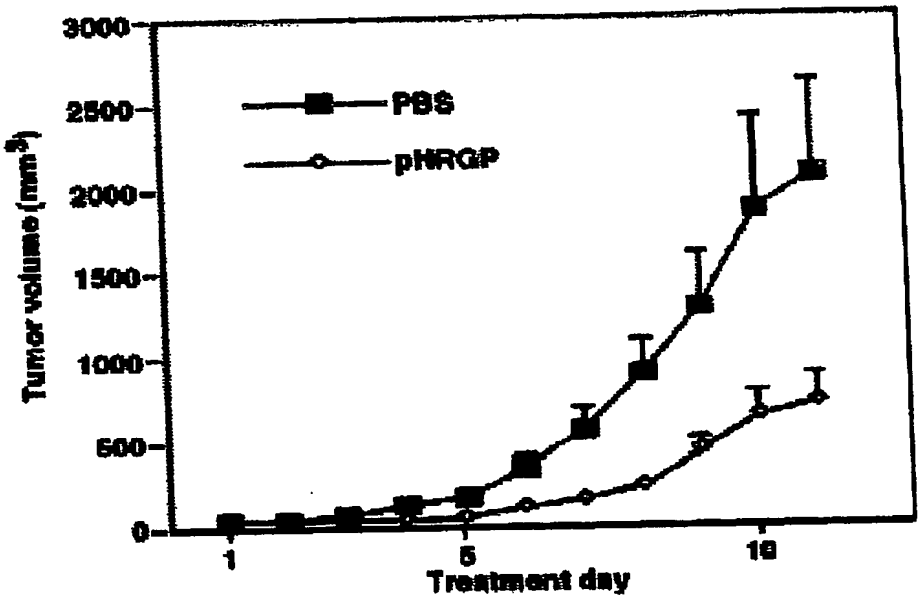

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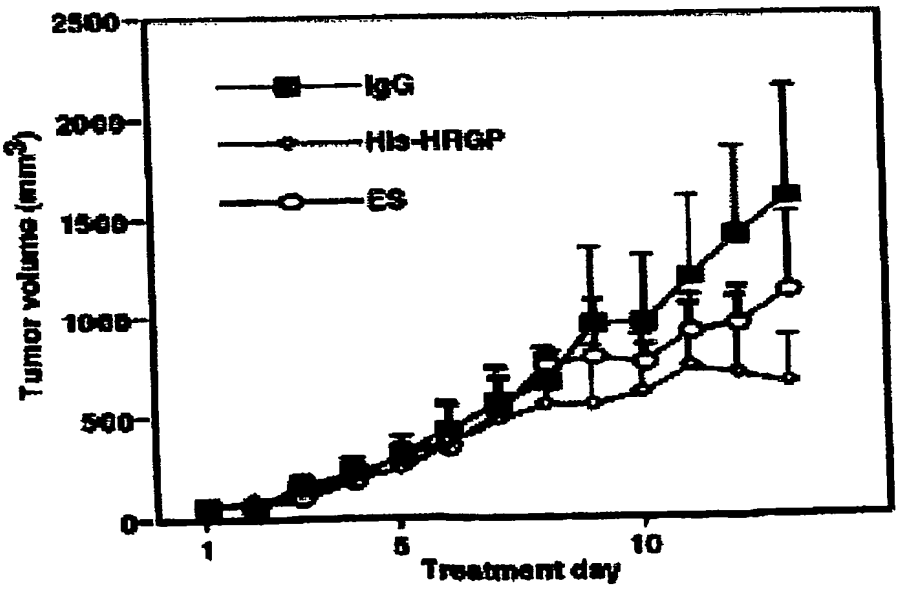

Fig 2

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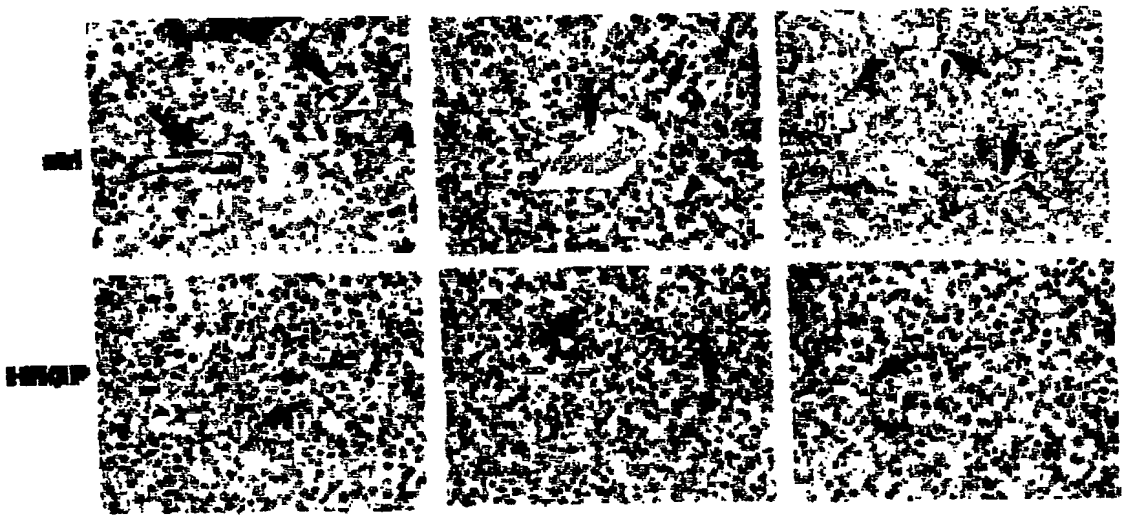
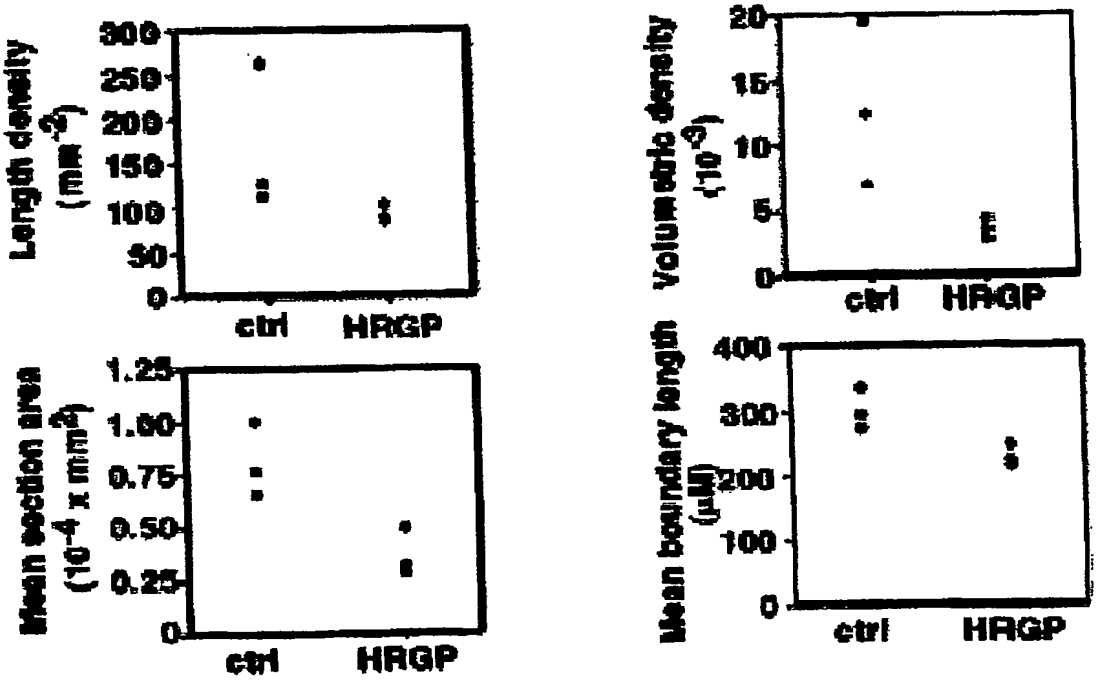

ctrl
HRGP

D: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300
250
200
150
100
50
0
ctrl
HRGP
Volumetric density (10-3)
20
15
10
5
0
ctrl
HRGP
Mean section area (10-4 x mm2)
1.25
1.00
0.75
0.50
0.25
0
ctrl
HRGP
Mean boundary length (µM)
400
300
200
100
0
ctrl
HRGP

Fig 2

D:

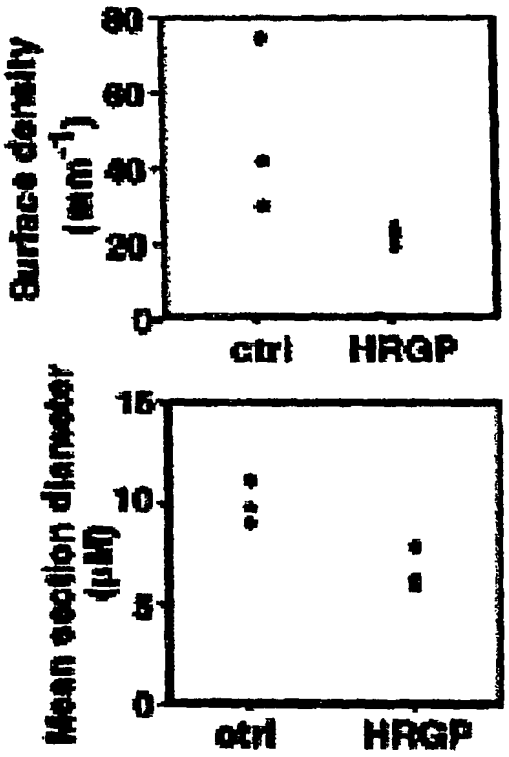
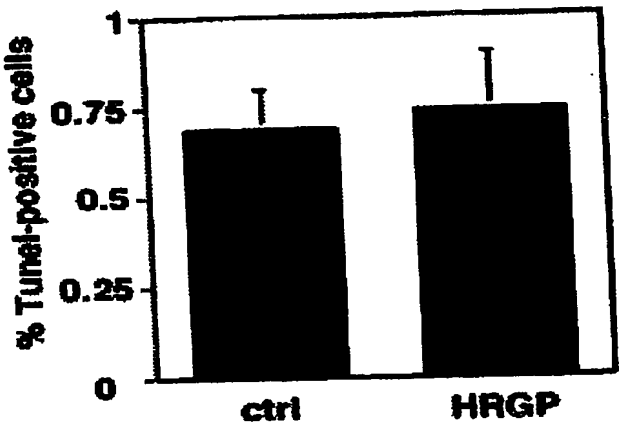

Surface density
(mm^{-1})
80
60
40
20
0
ctrl
HRGP
Mean section diameter
(µM)
15
10
5
0
ctrl
HRGP

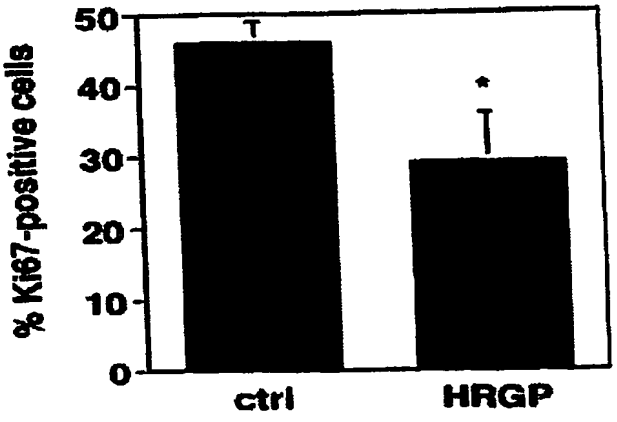
Fig 2

A: Apoptosis



TUNEL-staining

B: Proliferation



Ki67 staining

Fig 3

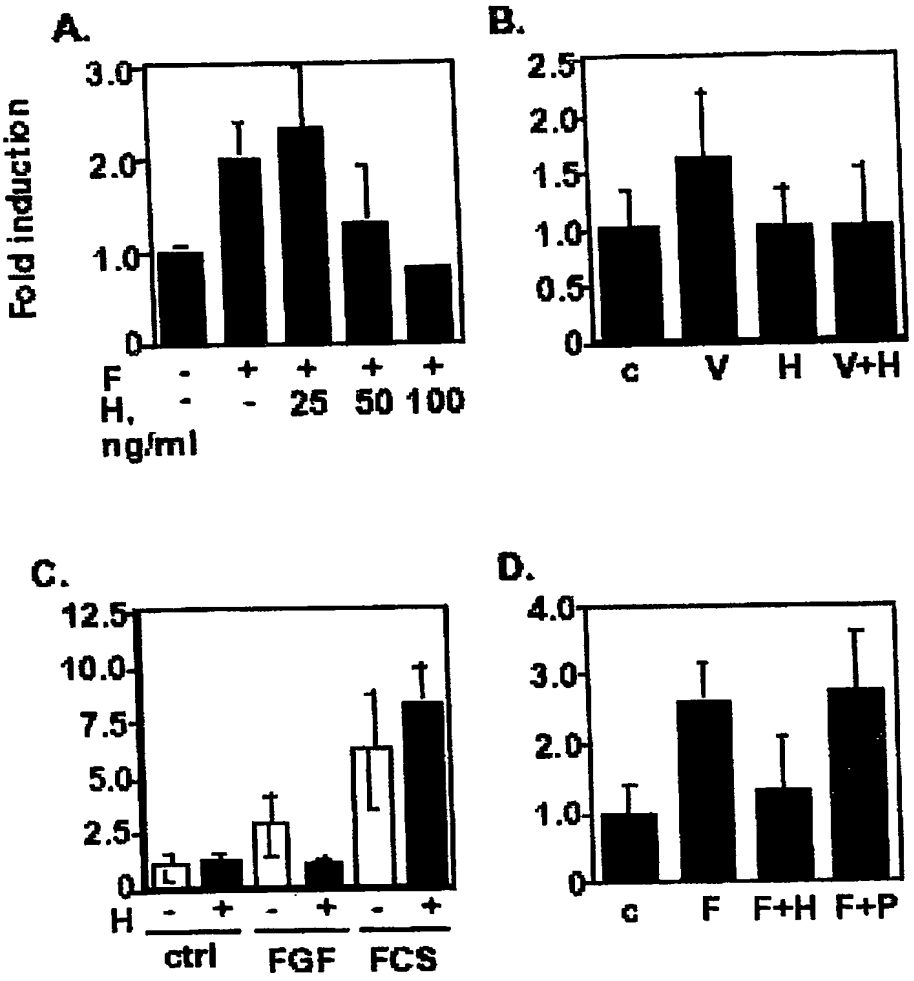

A.
Fold induction
3.0
2.0
1.0
0
F - + + + +
H, - - 25 50 100
ng/ml
B.
2.5
2.0
1.5
1.0
0.5
0
c V H V+H
C.
12.5
10.0
7.5
5.0
2.5
0
H - + - + - +
ctrl FGF FCS
D.
4.0
3.0
2.0
1.0
0
c F F+H F+P

Fig 4

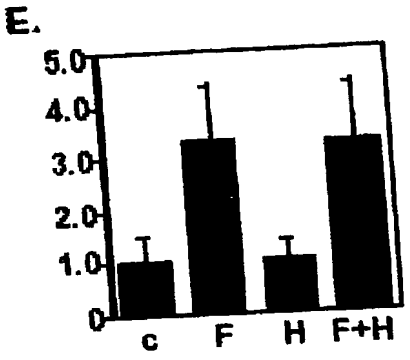


Fig 4

A:

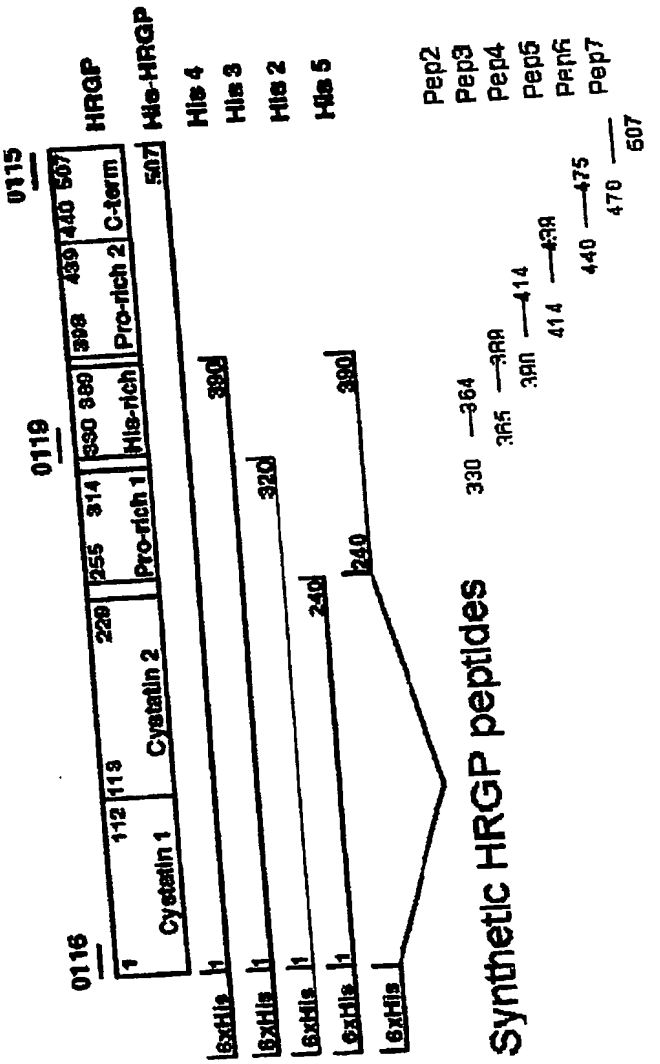


Fig 5

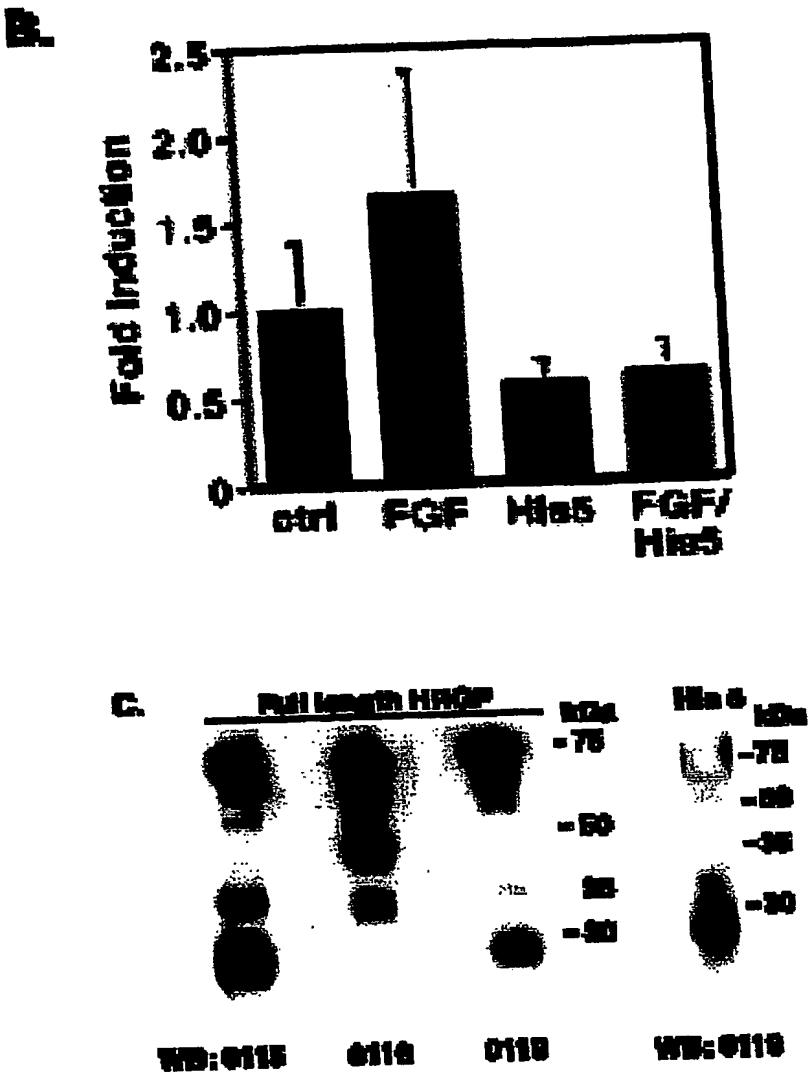


Fig 5

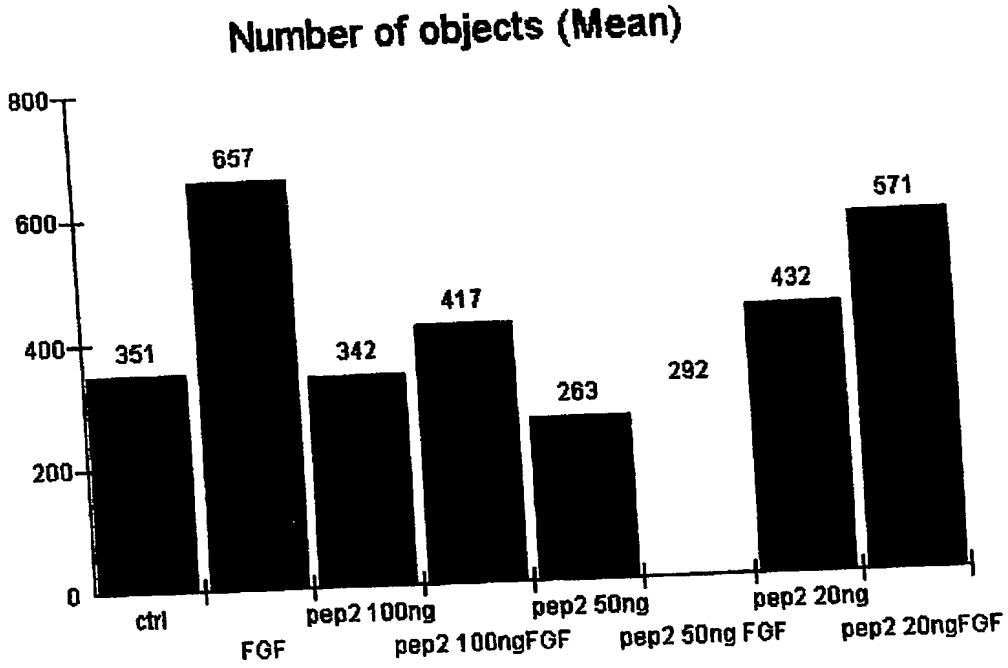


Fig 6

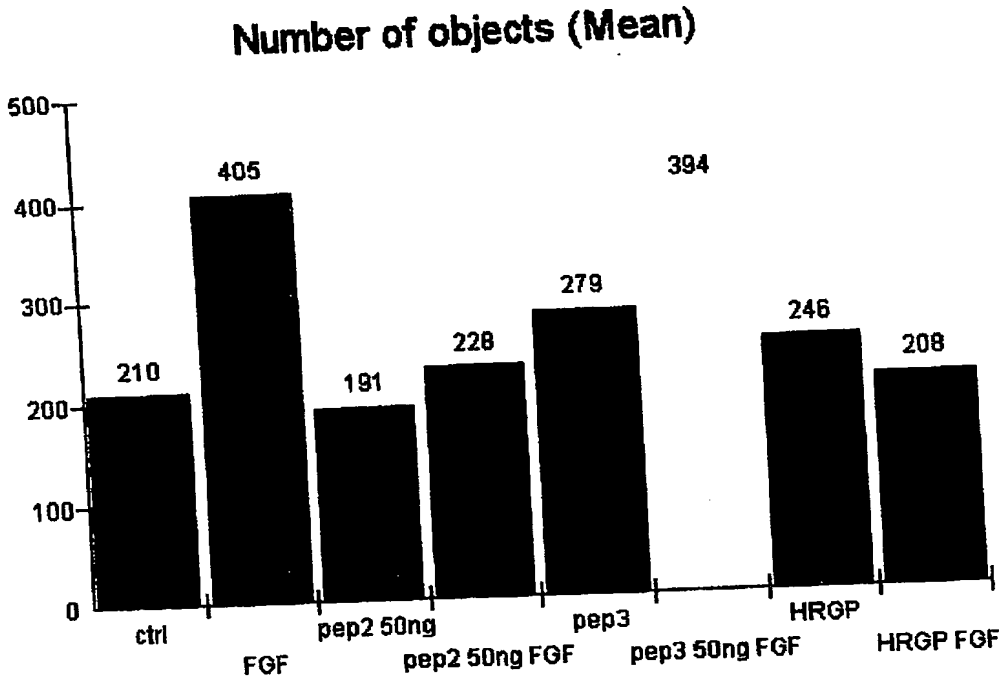
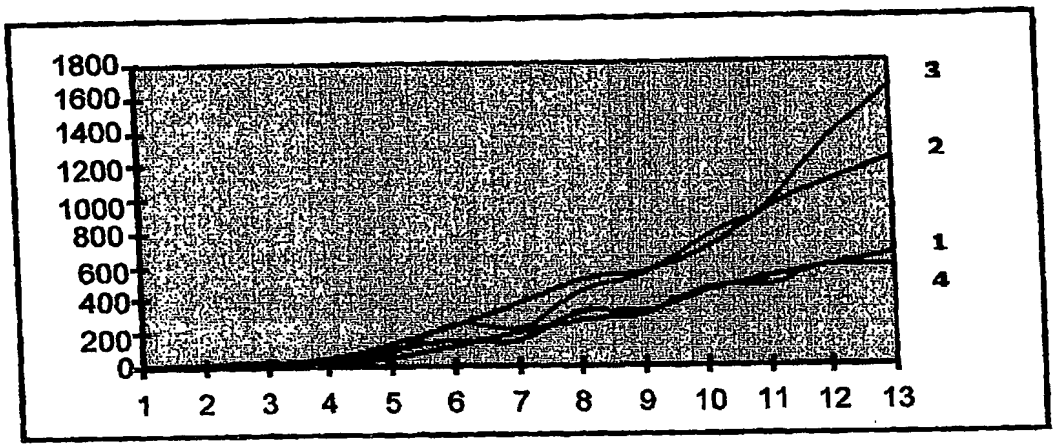

Number of objects (Mean)
500
400
300
200
100
0
210
405
191
228
279
394
246
208
ctrl
FGF
pep2 50ng
pep2 50ng FGF
pep3
pep3 50ng FGF
HRGP
HRGP FGF

Fig 7

A:



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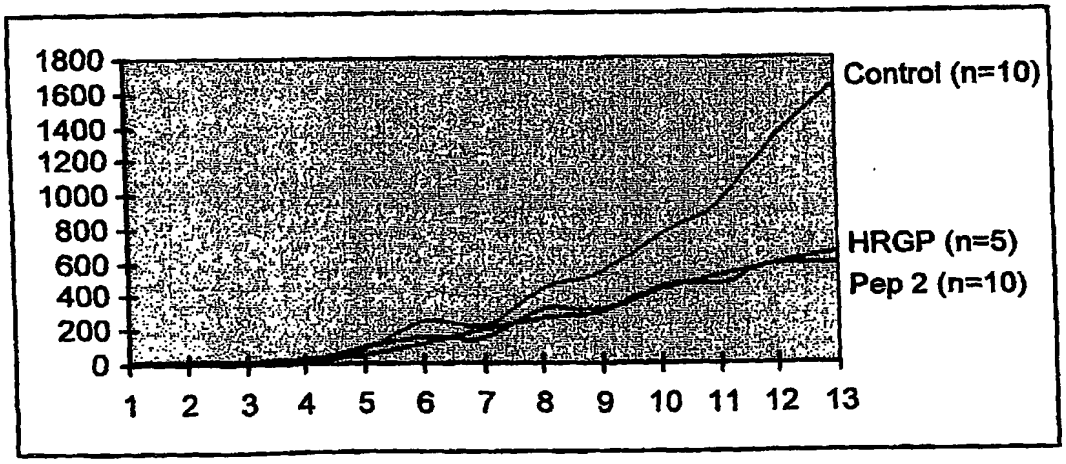

Fig 8

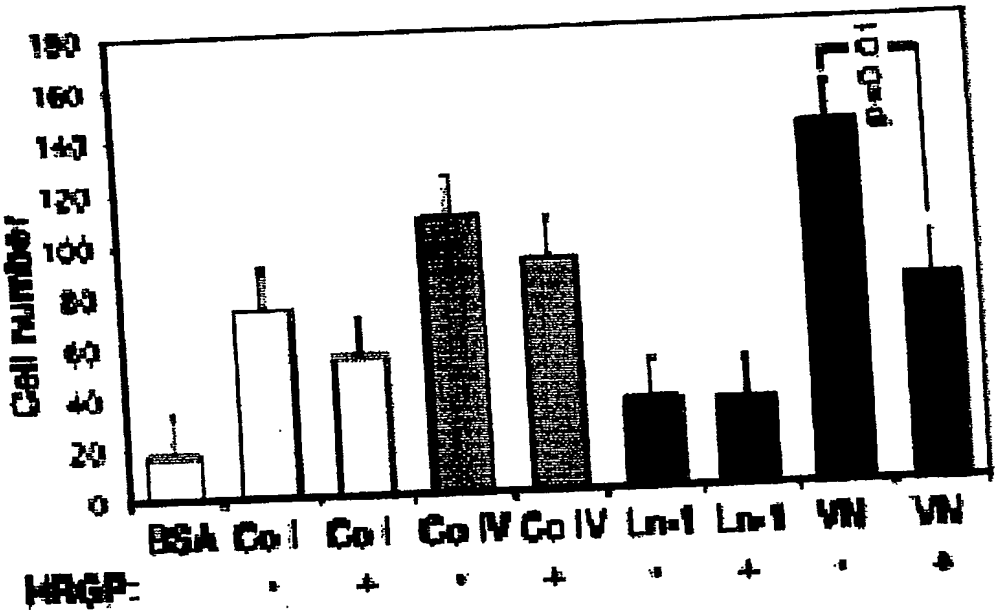

Fig 9

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